43408

National Library
of Canada

Bibliothèque nationale
du Canada

**CANADIAN THESES
ON MICROFICHE**

***THÈSES CANADIENNES
SUR MICROFICHE***

NAME OF AUTHOR/*NOM DE L'AUTEUR* GARY J. GIBSON

TITLE OF THESIS/*TITRE DE LA THÈSE* PROTEOGLYCANS OF THE BOVINE PERIODONTAL LIGAMENT

UNIVERSITY/*UNIVERSITÉ* UNIVERSITY OF ALBERTA

DEGREE FOR WHICH THESIS WAS PRESENTED/ *GRADE POUR LEQUEL CETTE THÈSE FUT PRÉSENTÉE* Ph D

YEAR THIS DEGREE CONFERRED/*ANNÉE D'OBTENTION DE CE GRADE* 1979

NAME OF SUPERVISOR/*NOM DU DIRECTEUR DE THÈSE* H C. Pearson

Permission is hereby granted to the NATIONAL LIBRARY OF CANADA to microfilm this thesis and to lend or sell copies of the film.

The author reserves other publication rights, and neither the thesis nor extensive extracts from it may be printed or otherwise reproduced without the author's written permission.

L'autorisation est, par la présente, accordée à la BIBLIOTHÈQUE NATIONALE DU CANADA de microfilmer cette thèse et de prêter ou de vendre des exemplaires du film.

L'auteur se réserve les autres droits de publication; ni la thèse ni de longs extraits de celle-ci ne doivent être imprimés ou autrement reproduits sans l'autorisation écrite de l'auteur.

DATED/*DATÉ* 17th April 1979 SIGNED/*SIGNÉ* Gary J. Gibson

PERMANENT ADDRESS/*RÉSIDENCE FIXÉ* 41 Locksley Rd.
Rye Victoria
Australia

NL-91 (3-74)

National Library of Canada

Cataloguing Branch
Canadian Theses Division

Ottawa, Canada
K1A 0N4

NOTICE

The quality of this microfiche is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us a poor photocopy.

Previously copyrighted materials (journal articles, published tests, etc.) are not filmed.

Reproduction in full or in part of this film is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30. Please read the authorization forms which accompany this thesis.

THIS DISSERTATION
HAS BEEN MICROFILMED
EXACTLY AS RECEIVED

NL-339 (3/77)

Bibliothèque nationale du Canada

Direction du catalogage
Division des thèses canadiennes

AVIS

La qualité de cette microfiche dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de mauvaise qualité.

Les documents qui font déjà l'objet d'un droit d'auteur (articles de revue, examens publiés, etc.) ne sont pas microfilmés.

La reproduction, même partielle, de ce microfilm est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30. Veuillez prendre connaissance des formules d'autorisation qui accompagnent cette thèse.

LA THÈSE A ÉTÉ
MICROFILMÉE TELLE QUE
NOUS L'AVONS REÇUE

THE UNIVERSITY OF ALBERTA

PROTEOGLYCANS OF THE BOVINE PERIODONTAL LIGAMENT

by

© GARY J. GIBSON

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
OF DOCTORATE OF PHILOSOPHY
IN
FACULTY OF DENTISTRY
DIVISION OF ORAL BIOLOGY

EDMONTON, ALBERTA
SPRING, 1979

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read and recommended to the Faculty of Graduate Studies for acceptance, a thesis entitled "Proteoglycans of the Bovine Periodontal Ligament". Submitted by Gary J. Gibson in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

C. H. Pearson
....
Supervisor

....

Norman R Davis
....

....

....

....

....

Date January 3, 1979

ABSTRACT

The periodontal ligament of the mature bovine incisor was shown to contain 0.67% (dry weight) glycosaminoglycan. Using CPC precipitation and hyaluronidase digestion this was shown to be composed of 43% dermatan sulphate type glycosaminoglycan, 31% chondroitin sulphate type glycosaminoglycan, 24% hyaluronic acid, a small proportion of material tentatively identified as heparan sulphate and possibly some undersulphated glycosaminoglycan. Fractionation of the galactosaminoglycans by stepwise precipitation with ethanol gave four distinct species that varied in iduronic acid content. These were grouped into approximately equal proportions of two broad species of galactosaminoglycans, one of the chondroitin sulphate family, the other of the dermatan sulphate family. Hyaluronidase digestion and periodate oxidation-alkali cleavage showed that the alcohol fractions contained copolymeric galactosaminoglycans composed of varying proportions of glucuronic and iduronic acids.

All the alcohol fractions with the exception of the smaller 50% alcohol fraction (molecular weight approximately 21,000) had very similar molecular weights (approximately 30,000) as shown by gel chromatography and end-group analysis. The alcohol fractionated galactosaminoglycans had low degrees of polydispersity and together with the galactosaminoglycans isolated from skin and cartilage proteoglycans exhibited very similar elution behaviour on Sephadex G-200.

Proteoglycans were extracted from the periodontal ligament by sequential treatment with 0.1 M NaCl, 2 M NaCl and 4 M guanidinium chloride and purified by DEAE-cellulose chromatography, density gradient centrifugation and gel chromatography. The proteoglycans extracted were characterized by composite agarose polyacrylamide gel electrophoresis, gel chromatography, analytical ultracentrifugation (in some cases) and chemical analysis.

The proteoglycans extracted with 0.1 M NaCl were predominantly of higher buoyant density, larger size and lower protein content than those extracted with 4 M guanidinium chloride. They appeared to contain a predominance of the chondroitin sulphate type glycosaminoglycans as well as some hyalūronic acid and an intermediate fraction possibly containing undersulphated glycosaminoglycan and/or heparan sulphate. Though heterogeneous on gel chromatography, they showed a resemblance in size and composition to the proteoglycans extracted from cartilage.

The proteoglycans extracted with 4 M guanidinium chloride were distinctly different from those extracted with 0.1 M NaCl. Physical and chemical characterization indicated a predominance of a single species of proteoglycan containing 47% protein and 53% of a dermatan sulphate type glycosaminoglycan. The proteoglycan has a molecular weight of approximately 130,000 and appears to contain an average of two dermatan sulphate chains (30,000 molecular weight) linked to a protein core of approximately 61,000 molecular weight. These proteoglycans showed striking similarity with a proteoglycan isolated from bovine skin using similar techniques; the only difference appears to be in the length of the glycosaminoglycan chain. The skin and periodontal ligament dermatan sulphate proteoglycans showed a tendency to aggregate and also appeared to contain a very closely associated protease. A tentative model for these dermatan sulphate proteoglycans is proposed.

A study of the glycosaminoglycan composition of the developing periodontal ligament showed that the hyaluronic content decreased dramatically very early in development, whereas the sulphated galactosaminoglycans increased with development (on a dry weight basis). Changes in the content of the dermatan sulphate like galactosaminoglycans (isolated by alcohol fractionation) approximately paralleled the changes observed in the collagen content of the ligament. The chondroitin sulphate like galactosaminoglycans (isolated by alcohol fractionation) increased to a maximum in newly erupted incisors and then decreased slightly in the mature occluded incisors. These changes are discussed in relation to the proposed functions of the associated proteoglycans.

ACKNOWLEDGEMENTS

I would like to acknowledge the assistance and encouragement of a number of friends and colleagues.

Dr. Harold Pearson, my supervisor, has always lent a sympathetic ear to any problems arising in the laboratory. He was always available with helpful advice and guidance, yet allowed me the freedom to plan my own work and make my own mistakes.

Dr. Dave Carmichael has provided sound advice and encouragement, both academic and non-academic.

Dr. Norm Davis has been an invaluable friend and has provided a great deal of encouragement and support, particularly during my first year in Edmonton.

I would also like to thank Drs. Paul Scott and Marisa Wohllebe for their helpful advice and encouragement.

Diane Davies performed the proteoglycan incubation experiments and subsequent composite agarose-polyacrylamide gel electrophoresis reported in this thesis. For this, and for helping to provide a friendly working atmosphere, I would like to express my sincere thanks.

I would like to thank Sophie Lehocky for assistance in the isolation of some of the skin proteoglycan preparations and along with Linda Ainsworth for performing the hexosamine analyses and general assistance around the laboratory.

I thank Dan Fackre for the amino acid analyses, Asif Rizvi and Dennis Carmel for the fine histology and Rosemary Fuller for secretarial assistance.

To two friends, John Cavanagh and Brian Banks, who were the source of considerable technical help and whose enthusiasm for non-academic pursuits made my stay in Edmonton enjoyable, I express my thanks.

The small team of friends responsible for the technical preparation of this thesis merit special thanks. Mrs. Hollingsworth typed the early drafts and her enthusiasm and energy were undaunted though successive drafts kept reappearing in virtually unrecognizable form. Vikki Scott not only fastidiously typed the final draft but improved my punctuation and English and proof read as she went. Sandi Gibson, as well as putting up with an oft-times irascible husband, made my handwriting almost legible, proof read each draft and organized the references.

TABLE OF CONTENTS

CHAPTER PAGE

1 INTRODUCTION 1
1.1 CONNECTIVE TISSUES 1
1.2 THE PERIODONTAL LIGAMENT 2
1.2.1 Structure 2
1.2.2 Development 4
1.2.3 Functions 6
1.2.4 Collagen 9
1.2.5 Glycosaminoglycans 11
1.3 PROTEOGLYCAN STRUCTURE 12
1.3.1 Glycosaminoglycans 12
1.3.1.1 Hyaluronic Acid 14
1.3.1.2 Chondroitin Sulphate 14
1.3.1.3 Dermatan Sulphate 17
1.3.1.4 Heparan Sulphate and Heparin 18
1.3.1.5 Keratan Sulphate 19
1.3.2 Cartilage Proteoglycans 21
1.3.3 Proteoglycans of Tissues other than Cartilage 25
1.4 THE PHYSIOLOGICAL FUNCTIONS OF PROTEOGLYCANS 28
1.5 AIMS OF THE PRESENT INVESTIGATION 33

2 MATERIAL AND METHODS 34
2.1 MATERIALS 34
2.2 METHODS 36
2.2.1 Isolation and Fractionation of Glycosaminoglycans 36
2.2.1.1 Extraction 36
2.2.1.2 CPC Precipitation of Glycosaminoglycans 36
2.2.1.3 Ethanol Precipitation of Glycosaminoglycans 38
2.2.2 Enzymic and Chemical Digestion of the Glycosaminoglycans 39

CHAPTER PAGE

2.2.2.1 Hyaluronidase Digestion 39

2.2.2.2 Chondroitin ABC Lyase Digestion 40

2.2.2.3 Periodate Oxidation and Alkaline Degradation 40

2.2.2.4 Digestion with Nitrous Acid 41

2.2.3 Molecular Weight Determination of Glycosaminoglycans by End Group Estimation Using Tritiated Sodium Borohydride Reduction 41

2.2.3.1 Alkaline Cleavage and Borohydride Reduction 41

2.2.3.2 Uronic Acid Content of Glycosaminoglycan Samples 42

2.2.3.3 Standardization of Tritiated Sodium Borohydride 43

2.2.3.4 Calculation of Molecular Weight 44

2.2.4 Gel Chromatography of Glycosaminoglycans 44

2.2.5 Cellulose Acetate Electrophoresis of Glycosaminoglycans 45

2.2.6 Isolation and Purification of Proteoglycans 45

2.2.6.1 Cartilage Proteoglycans 45

2.2.6.2 Non Cartilaginous Proteoglycans 47

2.2.6.2.1 Extraction 47

2.2.6.2.2 Purification by DEAE-cellulose Chromatography 49

2.2.6.2.3 Density Gradient Purification 50

2.2.7 Protease Digestion of Skin and Ligament Proteoglycans 50

2.2.8 Incubation of Highly Purified Proteoglycans at 37° C 51

2.2.9 Gel Chromatography of Proteoglycans 51

2.2.10 Composite Polyacrylamide-Agarose Gel Electrophoresis 51

2.2.10.1 Gel Preparation 52

2.2.10.2 Electrophoresis Conditions 52

2.2.10.3 Localization of Bands 52

CHAPTER PAGE

2.2.11 Determination of Molecular Weights by Sedimentation Equilibrium 53
2.2.12 Chemical Estimations 54
2.2.12.1 Collagen 54
2.2.12.2 Uronic Acid 54
2.2.12.3 Protein 54
2.2.12.4 Dermatan Sulphate and Iduronic Acid 54
2.2.12.5 Sulphate 56
2.2.12.6 Radioactivity 57
2.2.12.7 Hexosamines 57
2.2.12.8 Amino Acid Analyses 58
2.2.13 Histology 58

3 THE GLYCOSAMINOGLYCANS OF THE PERIODONTAL LIGAMENT OF THE MATURE OCCLUDED BOVINE INCISOR 59
3.1 GROSS AND HISTOLOGICAL APPEARANCE OF THE INCISOR PERIODONTAL LIGAMENT 59
3.2 TECHNIQUES OF GLYCOSAMINOGLYCAN ANALYSIS IN GENERAL 59
3.3 ANALYSIS OF THE PERIODONTAL LIGAMENT GLYCOSAMINOGLYCANS FRACTIONATED BY CPC PRECIPITATION 60
3.4 ANALYSIS OF THE PERIODONTAL LIGAMENT GALACTOSAMINOGLYCANS FRACTIONATED BY ALCOHOL PRECIPITATION 68
3.4.1 Chemical Analysis 68
3.4.2 Gel Chromatography 71
3.4.3 Molecular Weight Determinations 74
3.4.4 The Copolymeric Nature of Ligament Glycosaminoglycans 84

4 THE PROTEOGLYCANS OF THE PERIODONTAL LIGAMENT 96
4.1 INTRODUCTION 96
4.2 EXTRACTION 97
4.2.1 Extraction of Bovine Skin with Guanidinium Chloride Solutions 97

CHAPTER PAGE

4.2.2 Sequential Extraction of the Periodontal Ligament 97

4.3 PURIFICATION AND CHARACTERIZATION 100

4.3.1 DEAE-cellulose Chromatography 100

4.3.2 Density Gradient Centrifugation of Skin and Ligament Proteoglycans 103

4.3.3 Composite Agarose-Polyacrylamide Gel Electrophoresis of Density Gradient Fractions 112

4.3.4 Molecular Characteristics of the 4 M Guanidinium Chloride Extracted Proteoglycans 121

4.3.4.1 Gel Chromatography 121

4.3.4.2 Molecular Weight 127

4.3.4.3 Amino Acid Analysis 127

4.3.5 Molecular Characteristics of the 0.1 M NaCl Extracted Periodontal Ligament Proteoglycan 130

4.3.6 Glycosaminoglycan Composition of the Proteoglycans 133

4.4 THE DEGRADATION OF SKIN AND LIGAMENT PROTEOGLYCANS BY TISSUE PROTEINASES 139

4.5 ENDOGENOUS PROTEOLYTIC ENZYME AND THE AGGREGATION BEHAVIOUR OF HIGHLY PURIFIED SKIN AND LIGAMENT PROTEOGLYCANS 150

5 GLYCOSAMINOGLYCANS OF THE DEVELOPING BOVINE INCISOR PERIODONTAL LIGAMENT 156

5.1 GROSS APPEARANCE OF THE DEVELOPING BOVINE INCISOR PERIODONTAL LIGAMENT 156

5.2 HISTOLOGY OF THE DEVELOPING PERIODONTAL LIGAMENT 157

5.3 CHANGES IN THE COLLAGEN AND GLYCOSAMINOGLYCAN CONTENT WITH LIGAMENT DEVELOPMENT 163

5.4 CHANGES IN THE ALCOHOL FRACTIONATED GLYCOSAMINOGLYCANS WITH LIGAMENT DEVELOPMENT 163

6 DISCUSSION 175

6.1 PERIODONTAL LIGAMENT GLYCOSAMINOGLYCANS 175

6.1.1 Copolymeric Nature of the Periodontal Ligament Galactosaminoglycans 175

CHAPTER PAGE

6.1.2 The Molecular Weight of the Ligament Galactosaminoglycans and Their Behaviour on Gel Chromatography 182

6.2 THE PROTEOGLYCANS OF THE PERIODONTAL LIGAMENT 185

6.2.1 The Chondroitin Sulphate Proteoglycans 187

6.2.2 Dermatan Sulphate Proteoglycan 189

6.3 CHANGES IN THE LIGAMENT GLYCOSAMINOGLYCANS ASSOCIATED WITH TOOTH DEVELOPMENT 197

6.4 SUGGESTIONS FOR FURTHER WORK 202

BIBLIOGRAPHY 206

LIST OF TABLES

Table	Description	Page
1.	Genetically Distinct Types of Collagen	10
2.	Disaccharide Units in Glycosaminoglycans	15
3.	Occurrence and Properties of Glycosaminoglycans	16
4.	Bovine Incisor Classification	35
5.	The Uronic Acid Content of Galactosaminoglycan Samples	43
6.	Recovery of Standard Glycosaminoglycans after CPC Precipitation	61
7.	Recovery of Glycosaminoglycans after Hyaluronidase Digestion	62
8.	Glycosaminoglycan Composition of Bovine Periodontal Ligament from Mature Occluded Incisors	63
9.	Alcohol Fractionation of Standard Glycosaminoglycans	69
10.	Analyses of Glycosaminoglycan Fractions from the Periodontal Ligament of the Mature Occluded Bovine Incisor	70
11.	Molecular Weight Values for Bovine Periodontal Ligament, Skin and Cartilage Glycosaminoglycans	84
12.	Analysis of the Peaks Obtained from Sephadex G-50 Chromatography of Hyaluronidase Digested Ligament Glycosaminoglycan Fractions	94
13.	Yield of Proteoglycan(s) from Bovine Skin After Sequential Guanidinium Chloride Extraction	98
14.	Yield of Proteoglycans and Collagen From Bovine Periodontal Ligament	99
15.	Recovery of Uronic Acid and Hydroxyproline after DEAE-cellulose Chromatography of the Tissue Extracts	102
16.	Analyses of the Fractions from the Density Gradient Centrifugation of Skin and Ligament Proteoglycans	111
17.	Gel Electrophoretic Properties of Skin and Ligament Density Gradient Fractions	119
18.	Amino Acid Composition of Dermatan Sulphate Proteoglycans	128
19.	Amino Acid Composition of Chondroitin Sulphate Proteoglycans	134
20.	Glycosaminoglycan Composition of Bovine Skin, Ligament and Cartilage Proteoglycans	137

Table	Description	Page
21.	Gel Electrophoretic Properties of Skin and Ligament Proteoglycans and Digestion Products	148
22.	Gel Electrophoretic Properties of Incubated Skin and Ligament Proteoglycans	153
23.	Composition of the Alcohol Fractionated Glycosaminoglycans From the Developing Periodontal Ligament	172

LIST OF FIGURES

Figure		Page
1.	Schematic Representation of the Sagittal Section of an Incisor	3
2.	The Repeating Disaccharide Structure of the Glycosaminoglycans	13
3.	The Sequence of Polymer Modification Reactions Leading to Formation of the Predominant Disaccharide Unit in Heparin	20
4.	Schematic Model for the Structure of Cartilage Proteoglycans and the Interactions Involved in the Formation of Aggregates	23
5.	CPC Precipitation of Ligament Glycosaminoglycans	37
6.	Preparation of Aggregated and Disaggregated Cartilage Proteoglycans	46
7.	Extraction of Bovine Skin and Periodontal Ligament	48
8.	Densitometric Scans of Cellulose Acetate Electrophoresis of Ligament Glycosaminoglycans	66
9.	Gel Chromatography of the Alcohol Fractions of Ligament Glycosaminoglycans on Sephadex G-200	72
10.	Alkaline β Elimination and $NaBH_4$ Reduction of the Linkage Region of the Galactosaminoglycans	75
11.	Gel Chromatography on Sephadex G-200 of End-Labelled Glycosaminoglycan Fractions Obtained from Proteoglycans by Alkaline Cleavage and Reduction with Tritiated Borohydride	77
12.	Fractionation Coefficient (Kav; Laurent and Killander, 1964) between Sephadex G-200 and Buffer for a Series of Glycosaminoglycan Fractions Derived from Bovine Nasal Cartilage Proteoglycan, Periodontal Ligament Proteoglycans and Bovine Skin Proteoglycan as a Function of Molecular Weight	82
13.	The Effect of pH on the Periodate Oxidation and Alkaline Elimination of Chondroitin Sulphate	86
14.	Gel Chromatography on Sephadex G-200 of the 40 and 50% Alcohol Fractions after Periodate Oxidation and Alkali Elimination	87
15.	Chromatography on Sephadex G-50 of the Periodate Oxidized - Alkali Cleaved and Hyaluronidase Digested, Ligament Glycosaminoglycan, Fractions	90
16.	Gel Chromatography of Hyaluronidase Digested 18% and 25% Alcohol Fractions of Periodontal Ligament Glycosaminoglycans on Sephadex G-200	93

Figure		Page
17.	Chromatography of the 4 M Guanidinium Chloride Extract on DEAE-cellulose	101
18.	Density Gradient Centrifugation of Skin Proteoglycan	104
19.	Density Gradient Centrifugation of Skin and Ligament Proteoglycans	106
20.	Electrophoretic Patterns of Glycosaminoglycans from the Density Gradient Fractions of Skin and Ligament Proteoglycans	109
21.	Densitometric Scans of Agarose-Polyacrylamide Gel Electrophoresis of Skin Proteoglycan Density Gradient Fractions	113
22.	Densitometric Scans of Agarose-Polyacrylamide Gel Electrophoresis of 4 M Guanidinium Chloride Extracted Ligament Proteoglycan Density Gradient Fractions	115
23.	Densitometric Scans of Agarose-Polyacrylamide Gel Electrophoresis of 0.1 M NaCl Extracted Ligament Proteoglycans	117
24.	Gel Chromatography of Skin Proteoglycan	122
25.	Gel Chromatography of 4 M Guanidinium Chloride Extracted Periodontal Ligament Proteoglycans	125
26.	Gel Chromatography of the 0.1 M NaCl Extracted Periodontal Ligament Proteoglycans	131
27.	Composite Gel Electrophoresis of Tissue Proteinase Digests of Skin Proteoglycan	140
28.	Composite Gel Electrophoresis of Tissue Protease Digests of 4 M Guanidinium Chloride Extracted Ligament Proteoglycan	143
29.	Composite Gel Electrophoresis of Tissue Protease Digests of 0.1 M NaCl Extracted Ligament Proteoglycan	146
30.	Gel Electrophoresis of Incubated Skin and Ligament Proteoglycans	151
31.	Changes in the Collagen and Glycosaminoglycan Composition of the Periodontal Ligament Associated with Development	164
32.	Changes in the Alcohol Fractions of the Ligament Glycosaminoglycans with Tooth Development	166
33.	Changes in the Chondroitin Sulphate-like and Dermatan Sulphate-like Copolymeric Glycosaminoglycans with Ligament Development	169
34.	Schematic Models of Some Possible Hybrid Structures of the Periodontal Ligament Galactosaminoglycans	177

Figure Page

35. The Molecular Weights of Two Series of Chondroitin Sulphate Fractions as a Function of Their Kav 183

36. Schematic Model of Dermatan Sulphate Proteoglycan 194

LIST OF PHOTOGRAPHIC PLATES

Plate	Description	Page
1.	Cellulose Acetate Electrophoresis of the Galactosaminoglycan Fractions of the Mature Incisor Periodontal Ligament	64
2.	Histology of Group 1 and 2 Bovine Incisors	158
3.	Histology of Groups 3, 4, 5, and 6 Bovine Incisors	160

LIST OF ABBREVIATIONS

CB	Coomassie blue
CPC	cetylpyridium chloride
CS	chondroitin sulphate
CTAB	cetyltrimethylammonium bromide
DEAE - cellulose	diethylaminoethyl-cellulose
DS	dermatan sulphate
EDTA	ethylene diamine tetra-acetic acid
EtOH	ethanol
Frn	fraction
GAG	glycosaminoglycan
$GlNH_2$	glucosamine
GUA	glucuronic acid
GuCl	guanidinium chloride
HA	hyaluronic acid
HA'se	hyaluronidase
$HexNH_2$	hexosamine
hrs.	hours
i.d.	internal diameter
IdUA	iduronic acid
$\overline{M}n$	number average molecular weight
$\overline{M}w$	weight average molecular weight
Mwt	molecular weight
PG	proteoglycan
PGS	proteoglycan subunit
PL	periodontal ligament
PMSF	phenylmethylsulphonyl fluoride
res.	residue
SDS	sodium dodecylsulphate
TB	toluidine blue
UA	uronic acid
Vo	void volume
Vt	total volume

CHAPTER 1

INTRODUCTION

1.1 CONNECTIVE TISSUES

The periodontal ligament is one of a broad variety of connective tissues distinguished by the types, concentrations and organization of material in their extracellular matrix. The extracellular material generally may be described as a three-dimensional collagen fibre network of varying flexibility embedded in a gel-like matrix of ground substance. However there is, in fact, no generalized connective tissue but a large number of highly specialized forms such as bone, cartilage, synovial fluid, intervertebral disk, skin, vessel walls, tendon, ligament, cornea and the vitreous body. In general, these tissues contain low cell populations. The metabolism of the cells seems to be concerned mainly with the biosynthesis and secretion of matrix material. It follows that many physiological functions of such tissues are determined largely by the nature and organization of the extracellular components. These components form the milieu for the cells they surround. They presumably filter and censor the molecular or physical information that reaches the cells and ultimately influence their function.

Investigations on connective tissues and their extracellular macromolecular components have been intensive for the past twenty years or so. A great deal of information has been gained on the physico-chemical properties, structures and metabolism and new knowledge of these areas is continually growing.

Collagen, the broad class of proteoglycans and the structural glycoproteins, might be considered the common macromolecular determinants of the connective tissues. This thesis concerns the study of one species of connective tissue component, the proteoglycans, in a single very specialized connective tissue, the periodontal ligament.

1.2 THE PERIODONTAL LIGAMENT

1.2.1 Structure

The periodontal ligament is a band of dense connective tissue situated between the cement of the tooth and the cribriform plate of the alveolar bone. In histological sections of mature human teeth, orientated bundles of collagen fibres, the principal fibres, appear to be the predominant constituents. Between these are collections of loose, less fibrous, connective tissue with which blood vessels are associated. Single fibres do not span the whole distance between bone and cement but rather make up bundles of fibres which interlace with each other and with the abundant finer interstitial groups (Shackleford, 1971). Fibres are seen to embed in both the cement and the alveolar bone. The embedded parts of the fibres are termed Sharpey's fibres.

The bundles of fibres are arranged in groups having varying orientation. Cervically, the alveolar crest fibres run from the crest of the alveolar process coronally toward the cement. Slightly nearer the root apex an adjacent group runs horizontally, while apically to this most of the bundles are seen to run obliquely from the alveolar bone in an apical direction (Fig.1).

As well as collagen fibres, the periodontal ligament contains argyrophilic and oxytalan fibres (thought to be an immature form of elastic fibres), though, in man at least, there are few or no elastic fibres except in the blood-vessel walls. There is general agreement that oxytalan fibres are embedded in cement at one end and run axially through the periodontal space, often to terminate in a blood-vessel wall (Fullmer, 1967, Carmichael, 1968).

The periodontal ligament possesses a rich blood supply (Saunders and Röckert, 1967) comprised of vessels that enter the periodontal space through the alveolar bone and also via the gingival and apical vessels and drain to the basket-like venous retia that are present in the apical part of the periodontal space and to a venous network passing through the alveolar bone. The periodontal ligament also has rich somatic efferent and autonomic afferent nerve supplies (Anderson *et al*, 1970).

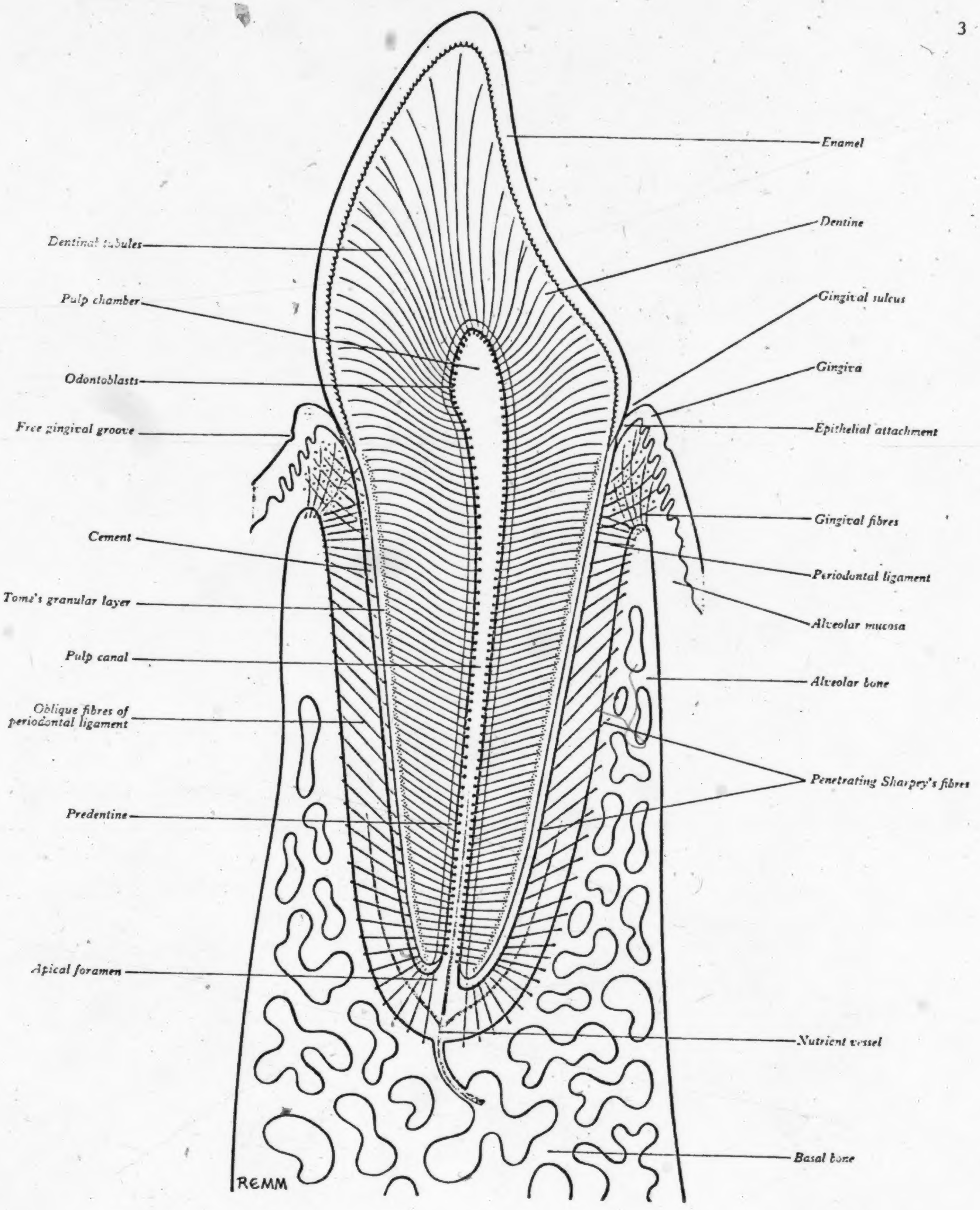

Enamel
Dentinal tubules
Dentine
Pulp chamber
Gingival sulcus
Odontoblasts
Gingiva
Free gingival groove
Epithelial attachment
Gingival fibres
Cement
Periodontal ligament
Tome's granular layer
Alveolar mucosa
Pulp canal
Alveolar bone
Oblique fibres of periodontal ligament
Penetrating Sharpey's fibres
Predentine
Apical foramen
Nutrient vessel
Basal bone
REMM

Fig. 1 Schematic Representation of the Sagittal Section of an Incisor.

The typical cells of the periodontal ligament are fibroblasts, undifferentiated mesenchyme cells, histiocytes, mast cells and epithelial cells (the rests of Malassez) that exist as a network close to the root surface (Sicher and Bhaskar, 1972).

1.2.2 Development

It has been well established that the periodontal ligament and cement are derived from the dental follicle. However confusion has arisen over different use of the term dental follicle. In this dissertation, the dental follicle is considered to be the entire mesenchymal layer between the developing tooth germ and the forming alveolar bone (Scott and Symons, 1971). It consists of three zones, an inner zone related to the tooth, an outer zone related to the alveolar bone and between the two an intermediate zone (Sicher and Bhaskar,1972). The layer of tissue immediately in contact with the tooth germ, the investing layer, appears to be the part of the dental follicle that gives rise to the cement and periodontal ligament (Freeman et al, 1975).

Root formation commences with epithelial proliferation at the cervical loop of the dental organ. This leads to the formation of Hertwig's epithelial root sheath, which seems to organize the differentiation of the odontoblasts underlying it. This complete, it becomes slightly separated from the newly formed root surface and fragments to form the epithelial cell rests of Malassez. The cement forming cells differentiate from the connective tissue collecting between the cell rests and the root surface. As the root sheath disintegrates collagen fibres are found between the separated epithelial cells and between these cells and the dentine surface. Following the migration of the epithelial cells away from the dentine surface undifferentiated cells are found adjacent to the root surface and these now become identifiable as cementoblasts.The fibrils near the tooth surface gradually become more numerous and deposition of crystallites occurs between the fibrils to form the first layer of cement. This first formed cement layer does not appear to contribute to the support of the tooth as Sharpey's fibres are not found within it. A layer of fibrous tissue lies against the outer surface of the epithelial debris and is aligned parallel to the root surface. With development there is an increased separation of the cell rests and fibrous layer from the tooth surface, with a corresponding increase in the amount of intervening connective tissue. Apart from the longitudinally arranged layer of collagen fibres, which appears to increase in density as it is traced occlusally, there is no discernible orientation of the cells or fibres in the remaining connective tissue.

With subsequent development fibroblasts on the cement surface lay down fine Sharpey's fibres. These fine thread-like structures are closely spaced and more prominent toward the cemento-enamel junction. Sharpey's fibres are subsequently seen emerging from the alveolar bone, extending into the periodontal space and then branching, to end in a splayed border. The central region of the periodontal ligament is still occupied by a loose collagenous element aligned parallel to the root surface. At about the stage of tooth eruption into the oral cavity, the fibres emanating from the alveolar bone appear to elongate into the central zone of the ligament and interdigitate with the lengthening cemental fibres near the cemento-enamel junction. Some of the fibre bundles thus formed appear to have an oblique orientation across the periodontal space. With the onset of clinical occlusion and function the fibre bundles become thicker, present an appearance of continuity from bone to cement and occur the full length of the root. The tissue thus formed has the classical appearance of the mature periodontal ligament.

The above description of periodontal ligament development is taken largely from a detailed study of the developing permanent teeth of squirrel monkeys (Grant and Bernick, 1972). Although there are many studies compatible with this description (O'Brian et al, 1958, Ten Cate, 1969 and Mashouf and Engel, 1975) the timing of the development of the principal fibre bundles, particularly those of oblique orientation, has been the subject of some dispute. Thomas (1965) and Tongue (cited in Ten Cate, 1969) demonstrated in human, rodent and cat developing teeth an oblique orientation of periodontal ligament fibres concomitant with the onset of tooth eruption. Similarly tooth germs transplanted subcutaneously develop a periodontal ligament which has dense bundles of collagen fibres with the classical orientation, although these teeth are completely non-functional (Freeman et al, 1975 and Wigglesworth, 1978, personal communication).

1.2.3 Functions

The functions of the periodontal ligament include the formation of bone and cement, the supply of nutrients to these tissues and sensory functions associated with perception of the position of the teeth in relation to surrounding tissues and to extraneous matter such as food. However, the major function of the ligament is probably to support the tooth and to accommodate tooth movement.

The periodontal ligament supports the tooth against stresses of varying magnitude and applied in various directions during mastication, swallowing, speaking and other empty jaw movements. The total amount of force involved is considerable, not only during chewing but also during other jaw movements; swallowing, for example, occurs about forty times per waking hour (Cunningham, 1971) and the occlusal force is considerable as well as often being of long duration. When these forces are applied to teeth they cause movement within the bony socket to a degree determined by the support systems of the ligament (Parfitt, 1967).

The arrangement of the principal fibres of the periodontal ligament as seen histologically suggests that a tooth is simply slung in its socket, and that any stress on the tooth is transmitted through the fibres to their attachments in the alveolar bone. However, studies on the movement of teeth under applied forces of known magnitude and direction (Parfitt, 1967 and Wills et al, 1972) and the derivation of mathematical and physical models (Gabel, 1956 and Synge, 1933) have shown that the periodontal ligament consists of more than such a single simple system. The concensus of present opinion is that there are several visco-elastic systems, including a fibre system, a ground substance system and a blood system (Melcher and Walker, 1975).

The collagen fibres are slightly wavy and thus cannot be put under axial stress until some tooth movement has occurred (Gabel, 1956). However it is suggested that they do resist tangential stress and thus restrain the flow of the proteoglycan matrix so that both act as a cushion between bone and tooth (see discussion of proteoglycans, section 1.4).

A major role has been attributed to the proteoglycan matrix in the support of teeth (Parfitt, 1967). This belief is rational, however it is impossible to say just what this role is as virtually nothing is known about the structure of the periodontal ligament proteoglycans. Furthermore the role of the proteoglycans cannot be considered in isolation. Their physiological functions seem highly dependent upon their interaction with solvent (water) and other proteins and carbohydrates of the extracellular milieu (see section 1.4).

The part played by the circulation has received considerable attention by investigators interested in the manner in which the periodontal ligament supports the tooth. It is evident that a tooth at rest moves in its socket in response to the vascular pulse (Korber, 1970 and Lear et al, 1972) and that a pulse-wave can be detected during a rebound of a tooth deflected by light loading (Parfitt, 1967). Exactly what happens to the blood in the periodontal space before, during, and after loading has been speculated upon, however, there appears to be very little experimental information in this area.

Much remains to be determined about the precise nature of these systems, in particular the way in which they interact, but the results of their physical characteristics is that when a force is applied to a tooth there is an initial rapid movement of the tooth in its socket followed by a slower phase; when the force is released there is an initial rapid recovery followed by a slower recovery phase (Parfitt, 1967).

The involvement of the periodontal ligament in tooth eruption has been elegantly illustrated in a number of experiments involving root resectioning. However most of these experiments have been carried out on the continuously erupting rodent incisor and some caution should be exercised in extrapolating these findings to the eruption of teeth of limited eruption, particularly to the stages of eruption occurring within the jaw. If the periapical tissues of the incisor (i.e. Hertwig's epithelial root sheath, proliferating papilla cells and the apical portion of the periodontal ligament) are removed surgically, eruption of the distal portion of the root continues, with eventual exfoliation even though no further root formation takes place (Berkovitz and Thomas, 1969, Berkovitz, 1971 and Moxham and Berkovitz, 1974). These experiments largely eliminated the contributions from root formation and periapical vasculature and, since the ligament was the only tissue remaining in association with the erupting tooth fragment, indicated that the force of eruption is generated within the periodontal ligament.

It is still not known how a tractile force is generated within the tissue, but two possibilities have been suggested. The first involves the maturation and contraction of collagen fibres perhaps in association with the proteoglycan matrix (Thomas, 1976). This proposal was supported by the finding that the administration of lathyrogens (inhibitors of collagen cross linking) produced a reduction in the eruption rates of rodent incisors (Thomas, 1965 and 1976). However there is no evidence to suggest that collagen fibre contraction occurs with maturation *in vivo*. The second suggestion, which is biologically the most attractive, proposes that fibroblasts within the ligament are responsible for generating the eruptive force either by their contractility or locomotor action (Ness, 1967). Discovery of the presence of microfilaments within the cytoplasm of fibroblasts of the periodontal ligament added convincing support to this proposal (Beertsen *et al*, 1974). Microfilaments are thought to function as part of a contractile system which is involved in cell movements; for instance cell locomotion (Wessels *et al*, 1971). The possible motile character of ligament fibroblasts and their involvement in tooth eruption was further supported by the observed coronal migration of these cells at a rate comparable with that of eruption (Beertsen,1975). Thus Beertsen *et al* (1974) proposed that fibroblasts, by means of their cytoplasmic microfilament system, continuously migrate in an occlussal direction and by means of the meshwork of collagen fibrils inserted in the cement, draw the tooth axially.

1.2.4 Collagen

The periodontal ligament, as a soft connective tissue, is composed predominantly of collagen together with smaller amounts of proteoglycans and noncollagenous proteins. Collagen accounts for approximately 50% of the dry weight of the ligament (Paunio, 1969 and Guis et al, 1973) and was shown to contain both type I and type III collagens. Estimates based on the amounts of certain CNBr peptides indicated that about one-fifth of the collagen is type III, the remainder being type I (Butler et al, 1975).

Detailed chemical analysis of the individual α chains has now demonstrated that considerable differences exist between the collagens of different tissues (see E. Miller, 1976, for a review). At the present time four well characterized, genetically distinct types of collagen have been identified (Table 1) and it is likely that further types will be discovered. Type I collagen of skin, tendon, bone and dentine contains two identical chains (α1) and a chemically distinct third chain (α2) (Piez et al, 1963). Type II collagen was the first genetically distinct collagen demonstrated in mammals and has been shown to occur in hyaline cartilage (Miller and Matukas, 1969) and intervertebral disk (Eyre and Muir, 1974). Type III collagen occurs in aorta (Trelstad, 1974), dermis (Miller et al, 1971 and Epstein, 1974), and uterine leiomyomas (Chung and Miller, 1974) in addition to the periodontal ligament. The relative proportions of type III and type I collagens in dermis change dramatically as a function of fetal development. Type III and type I collagen are present in approximately equal amounts in young fetal dermis but the proportion of type III collagen falls to less than 20% of the total collagen in the newborn infant and remains at about the same level throughout life (Epstein, 1974). Basement membrane collagen has not been completely characterized but has been designated as type IV and contains three identical α chains (Kefalides, 1975). It is highly likely that basement membranes from different sources may be shown to be distinct as more detailed analyses become available.

Type	Molecular Composition	Tissue Distribution	Hydroxylysine Content Residues/1000	Carbohydrate (% Hydroxylysine glycosylated)
I	$[\alpha 1(1)]_2\alpha^2$	Dermis, tendon, bone, dentine	6 - 8	< 20
II	$[\alpha 1(11)]_3$	Hyaline cartilage intervertebral disks	20 - 25	50
III	$[\alpha 1(111)]_3$	Dermis, Cardiovascular system. Smaller amounts in many tissues.	6 - 8	15-20
IV	$[\alpha 1(IV)]_3$	Basement Membranes	60 - 70	80
Possibly Several	unknown	Basement Membranes		

Table 1. Genetically Distinct Types of Collagen

Taken from Bailey and Robins (1976).

The metabolic activity of collagen in rat molar periodontal tissues was measured by following the incorporation of ^{3}H-proline into hydroxyproline. The rate of collagen synthesis in the periodontal ligament was found to be twice as fast as in attached gingiva, four times as fast as in skin corium, and six times as fast as in alveolar bone. The rate of incorporation of proline into mature collagen is also much higher in the periodontal ligament. A comparison between the rates of incorporation of label into newly synthesized collagen and into mature collagen showed that newly synthesized collagen in the periodontal ligament (and also in the alveolar bone) was quantitatively converted into insoluble collagen, whereas conversion efficiencies of 50 and 33 percent respectively were found in attached gingiva and in skin. The half life of the turnover of mature collagen was found to be 1 day in the molar periodontal ligament whereas a half life of 15 days was determined for skin corium. Slightly longer turnover times (3 days) were found for erupting mandibular incisor ligaments (Sodek, 1977).

Studies of collagen synthesis in explants of isolated periodontal ligaments, in tissue culture, showed that collagen synthesis constituted only a small proportion of the total protein synthesis (Rossman et al, 1975 and Sodek et al, 1977), however, it appears that a relatively small number of cells survive in these explants and many of the surviving cells enter the cell cycle and thus probably direct much of their protein synthesis to duplicating cellular macromolecules (Brunette et al, 1976). Autoradiographic evidence suggests that synthesis of collagen occurs evenly across the width of the ligament (Beertsen and Everts, 1977). The analysis of the distribution of collagen phagocytosis, however, revealed that in the mid-region of the ligament the amount of phagocytosed collagen was much greater than that in areas adjacent to the tooth or alveolar compartment in the continuously erupting mouse incisor (Beertsen and Everts, 1977) suggesting that remodelling of collagen may occur in an intermediate area of the ligament in this tissue. However collagen phagocytosis is more or less randomly distributed in the periodontal ligament of functional mouse molars (Ten Cate et al, 1976) suggesting there is no preferred site of remodelling in teeth of limited eruption.

1.2.5 Glycosaminoglycans

Hyaluronic acid, chondroitin 4-sulphate, condroitin 6-sulphate, dermatan sulphate and heparan sulphate have all been identified in the periodontal ligament (Paunio, 1969 and Munemoto et al, 1970, Pearson et al, 1975). Munemoto et al suggest that the ligament contains about half as much hyaluronic acid as chondroitin sulphate which is in slightly higher concentration than the dermatan sulphate. However the absolute content of individual glycosaminoglycans could not be determined because the weight of the periodontal ligament itself was not determined. Pearson et al (1975) found that approximately a quarter of the dermatan sulphate in the ligaments of bovine molars could be extracted with 0.15 M NaCl while the remainder appears closely associated with the collagenous matrix. Further it appears that most of the chondroitin sulphates and hyaluronic acid can be extracted with 0.15 M NaCl. Autoradiographic evidence suggests that the sulphated glycosaminoglycans are synthesized evenly across the ligament and turn over rapidly (Baumhammers and Stallard, 1968).

1.3 PROTEOGLYCAN STRUCTURE

The particular characteristics of each type of connective tissue results from differences in the relative proportions of fibrillar proteins and other constituents, principally in the relative proportions of collagen and proteoglycan. The types of collagen and proteoglycan vary in different connective tissues and interactions between them will also affect the properties of the tissue.

Cartilage proteoglycans have been extensively studied, however little is known about the proteoglycans of noncartilaginous tissues other than their glycosaminoglycan structures.

1.3.1. Glycosaminoglycans

The glycosaminoglycans are characteristic components of vertebrate connective tissues. With the possible exception of hyaluronic acid, they do not occur as free polysaccharide chains in vivo, but are linked at the terminal reducing sugar residue to a protein molecule to form proteoglycans. The structures of the glycosaminoglycans are similar in that they may be generally described as linear polymers built of disaccharide repeat units consisting of hexosamine and hexuronic acid (with the exception of keratan sulphate, which has glucosamine and galactose). Earlier views of glycosaminoglycan structures generally presented an idealized or 'parent' type of structure with a continuous repeat of a single disaccharide unit. It is becoming increasingly evident, however, that this representation is too simple and that actual molecules rarely contain only a single type of disaccharide unit. However, it is nevertheless convenient to class each glycosaminoglycan as belonging to a 'family' of related structures dominated by a particular disaccharide unit: seven families of glycosaminoglycans are commonly found in vertebrate tissues. The structures of their dominant repeat disaccharide units are given in Fig. 2 and these together with other disaccharides known to occur within each family are shown in Table 2. Examples of their distribution

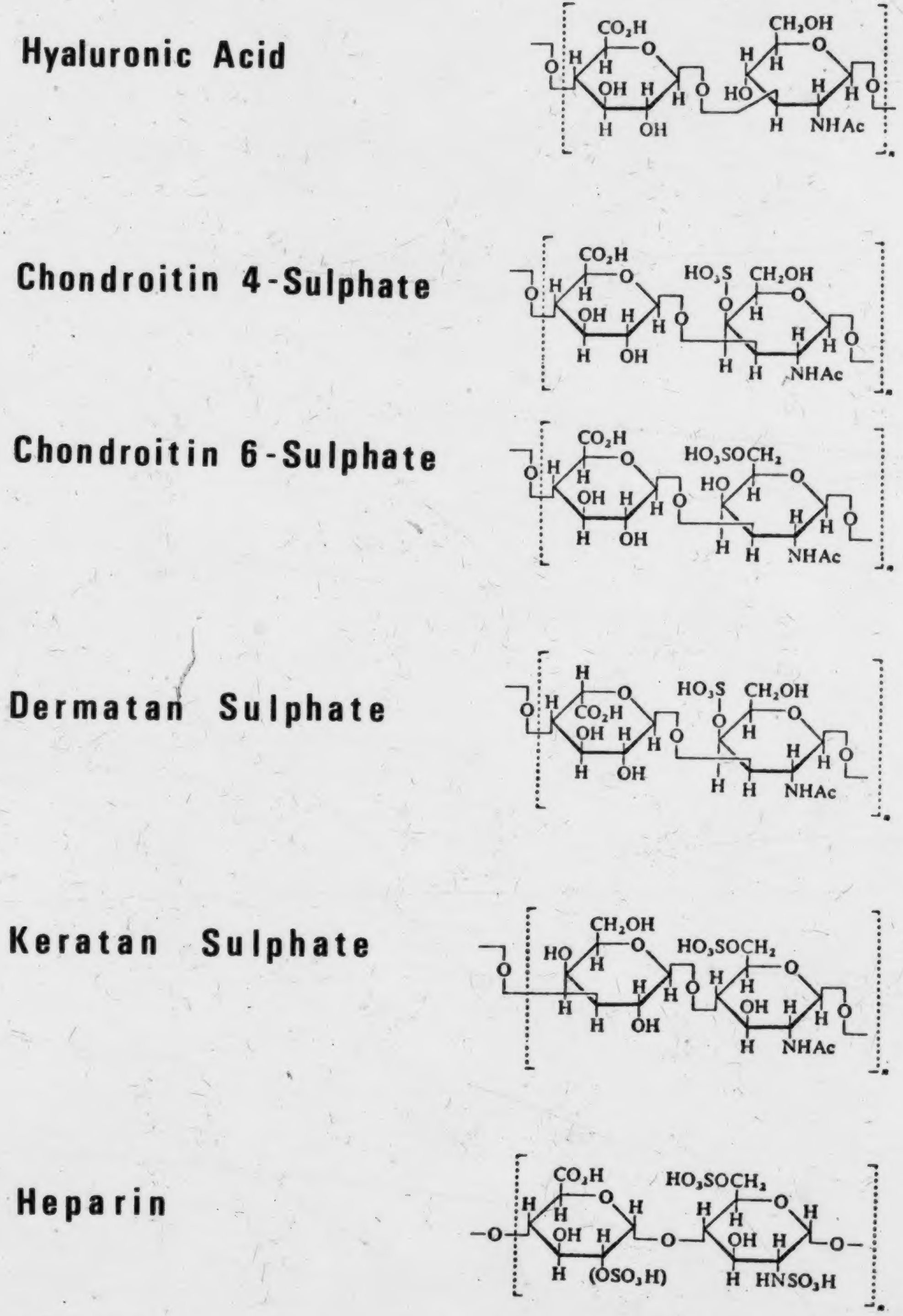

Hyaluronic Acid
CO₂H
CH₂OH
NHAc
Chondroitin 4-Sulphate
HO₃S
Chondroitin 6-Sulphate
HO₃SOCH₂
Dermatan Sulphate
Keratan Sulphate
Heparin
(OSO₃H)
HNSO₃H

Fig. 2 The Repeating Disaccharide Structure of the Glycosaminoglycans

and some physical data are given in Table 3.

1.3.1.1 Hyaluronic Acid

Hyaluronic acid is the only non-sulphated glycosaminoglycan and has a wide distribution in both vertebrate and invertebrate connective tissues. In mammals it is abundant in many embryonic tissues and it occurs in large amounts in synovial fluid, vitreous humour and umbilical cord. Hyaluronic acid does not occur as a multichain proteoglycan as is shown by its viscosity in solution being unaffected by proteolytic digestion (Ogston and Sherman, 1959). The question whether single chains are linked to protein remains in some doubt. Hyaluronic acid prepared by density gradient centrifugation still contains slightly less than 2% protein (Silpananta et al, 1968), though hyaluronic acid purified from synovial fluid by ion exchange chromatography was reported to contain only 0.35% protein (Scher and Hamerman, 1972). Proof of the covalent nature of the association of protein with hyaluronic acid awaits the isolation of a carbohydrate-protein linkage fragment.

1.3.1.2 Chondroitin Sulphate

Chondroitin sulphate occurs in very large amounts in all types of cartilage, where it can account for up to 40% of the dry weight of the tissue, but it also has a broad distribution in other connective tissues such as skin, cornea, blood vessel wall and nucleus pulposus. The disaccharide repeating unit has a structure similar to that of hyaluronic acid, but contains galactosamine instead of glucosamine and the galactosamine has an ester sulphate group attached to the 4 or 6 position. The molecular weight of chondroitin sulphate is in general between 5,000 and 50,000 and most preparations are polydisperse in size. The degree of sulphation can vary within a single preparation and from one tissue to another. Some sources, such as shark cartilage, characteristically contain oversulphated condroitin 6-sulphate (Suzuki, 1960) with extra sulphate on the uronic acid residue, whereas cornea contains an undersulphated form, referred to as chondroitin, which has

Polysaccharide	Sugar Residue 1	Sugar Residue 2	Sulphate Positions Sugar Res.1	 Sugar Res.2
	Dominant Disaccharide Repeat			
Hyaluronate	→3)βGlcNAc(1→4)	βGlcUA-(1→	0	0
Chondroitin 6-sulphate	→3)βGalNAc6SO_4(1→4)	βGlcUA-(1→*	a) 6	0
			b) 4+6	0
Chondroitin 4-sulphate	→3)βGalNAc4SO_4(1→4)	βGlcUA-(1→*	a) 4	0
			b) 4+6	0
Dermatan Sulphate	→3)βGalNAc4SO_4(1→4)	αIdUA-(1→*	a) 4	0
			b) 4	2 or 3
			c) 4+6	0
	(also contains →3)βGalNAc4SO_4(1→4)	βGlcUA-(1→*)	d) 4+6	2 or 3
			e) 0	2 or 3
Keratan Sulphate	→3)βGal(1→4)	βGlcNAc6SO_4-(1→*	a) 0	6
			b) 0	0
			c) 6	6
	Disaccharide Components			
Heparin like Polysaccharides	→4)αGlcNAc-(1→4)	αIdUA(1→	a) 0	2
			b) 6	0
			c) 6	2
	→4)αGlcNSO$_3$(1→4)	βGlcUA(1→	a) 6	0
	→4)αGlcNAc-(1→4)	βGlcUA(1→	a) 6	0
	→4)αGlcNSO$_3$(1→4)	αIdUA(1→	a) 6	0
			b) 6	2

Table 2. Disaccharide Units in Glycosaminoglycans

Although these structures represent most of the disaccharide units found in vertebrate glycosaminoglycans it is by no means comprehensive because the detailed structures of heparin like polysaccharides and keratan sulphate are still incomplete. SO_4, sulphate (ester); NSO_3, N-sulphamino; Gal, D-galactose; GlcUA, D-glucuronic acid; GlcNAc, N-acetyl-D-glucosamine; GalNAc, N-acetyl-D-galactosamine; IdUA, L-iduronic acid.
* the disaccharide sequences of chondroitin 4-sulphate, chondroitin 6-sulphate and dermatan sulphate are known to occur within the same chain. Taken largely from Comper and Laurent (1978).

Polysaccharide	Molecular Weight Range (x 10^{-3})	Charge per Disaccharide	Other Sugar Components	Examples of Occurrence in Mammalian Tissues
Hyaluronate	4000-8000	1.0		Ubiquitous in connective tissue (?) skin, synovial fluid, vitreous humor, heart valve, cartilage.
Chondroitin 4-sulphate	5-50	1.1 - 2.0	D-galactose D-xylose	Cartilage, cornea, bone, skin, arterial wall.
Chondroitin 6-sulphate	5-50	1.2 - 2.3	D-galactose D-xylose	Cartilage, bone, umbilical cord, intervertebral disk, heart valve.
Dermatan sulphate	15-40	2.0 - 2.2	D-galactose D-xylose	Skin, heart valve, fibrous cartilage.
Keratan sulphate	4-19	0.9 - 1.8	D-galactosamine D-mannose L-fucose Sialic acid	Cornea, cartilage, intervertebral disk.
Heparan sulphate		1.1 - 2.8	D-galactose D-xylose	Lung, liver, arterial wall, ubiquitous on cell surfaces (?)
Heparin	4-16	3-4	D-galactose D-xylose	Lung, liver, skin, intestinal mucosa (intracellular in mast cells).

Table 3. Occurrence and Properties of Glycosaminoglycans

See reviews, Mathews, 1975, Bettelheim, 1970, Lindahl, 1976, Comper and Laurent, 1978, and Muir and Hardingham, 1975, for references. Other sugar components refer to sugars other than those found in the repeat disaccharide (see Table 2).

a molar ratio of sulphate to hexosamine as low as 0.12 (Meyer et al, 1953). Individual chondroitin sulphate chains may contain sulphate groups in both the 4 and 6 positions and may also be exclusively 4-sulphated or 6-sulphated.

1.3.1.3 Dermatan Sulphate

Dermatan sulphate is an isomer of the chondroitin sulphates in which a large portion of the D-glucuronate residues are replaced by their C5 epimer L-iduronate. Considerable structural variability has been demonstrated. The D-glucuronic acid content may range from negligible amounts to more than 90% of the total uronic acid. Some preparations have a comparatively simple structure. For example, pig skin dermatan sulphate consists, essentially of iduronosyl-N-acetylgalactosaminyl 4-sulphate disaccharide units (Fransson and Rodén, 1967a). However, others are extremely complex with an extensively hybridized polysaccharide backbone and, in addition, both 4- and 6-O-sulphated galactosamine residues (Fransson and Havsmark, 1970). Oversulphated regions may be due to the occurrence of sulphated L-iduronate residues; sulphated glucuronate has not been detected (Suzuki, 1960 and Malmström and Fransson, 1971).

The distribution of uronic acid residues in dermatan sulphate was investigated by Fransson and co-workers by specifically degrading a number of dermatan sulphate preparations either at glucuronic acid residues, using hyaluronidase (Fransson and Rodén, 1967 a and b) or chondroitinase AC, (Fransson and Havsmark, 1970 and Fransson and Malmström, 1971) or at iduronic acid residues by periodate oxidation followed by Smith degradation or alkali cleavage (Fransson, 1974, Fransson and Carlstedt, 1974, Fransson et al, 1974, Cöster et al, 1975). The structural characterization of the resulting fragments afforded the following conclusions. A major fraction of both the D-glucuronic and the L-iduronic acid units in dermatan sulphate occurs in blocks composed of varying numbers of glucuronosyl-N-acetylgalactosamine and iduronosyl-N-acetylgalactosamine repeating disaccharide units, respectively. The relative proportions of the two types of blocks may vary considerably, as is evident from the fact that the D-glucuronic acid content of dermatan sulphate preparations ranges from negligible amounts to more than 90% of the total uronic acid (Habuchi et al, 1973 and Inoue and Iwasaki, 1976). Although each kind of block structure may occur anywhere along the polysaccharide chain, there is generally an over-representation of D-glucuronic acid units in the immediate vicinity of the carbohydrate-protein link region. In the transition zones between more extended blocks, glucuronic acid and iduronic acid containing sequences alternate at shorter intervals.

The use of the chondroitinases AC and ABC has also allowed studies on the distribution of sulphate groups in dermatan sulphate and has shown that both kinds of uronic acid may occur bound to either 4- or 6-sulphated N-acetylgalactosamine residues (Fransson and Havsmark, 1970, Fransson et al, 1974 and Fransson, 1968). However, there is a general tendency, particularly with increasing block size, towards prevalence of L-iduronosyl-N-acetylgalactosamine 4-sulphate and D-glucuronosyl-N-acetylgalactosamine 6-sulphate units.

The occurrence of a wide range of hybrid polysaccharides introduces a problem of nomenclature. Should glycosaminoglycans in which iduronic acid constitutes less than 20% of the total uronic acid be included in the chondroitin sulphate or dermatan sulphate families? Furthermore it appears likely that fibrous connective tissues contain a variety of hybrid glycosaminoglycans rather than distinct chondroitin sulphate and dermatan sulphate polysaccharides (Fransson and Rodén, 1967 a and b, Fransson and Havsmark, 1970, Habuchi et al, 1973 and Inoue and Iwasaki, 1976). For the purpose of discussion, the glycosaminoglycans containing a small proportion of iduronic acid will be grouped with the chondroitin sulphates in this thesis.

1.3.1.4 Heparan Sulphate and Heparin

Heparin and heparan sulphate contain glucosamine and uronic acid in their disaccharide repeating units (Fig. 2). The glucosamine residue is either N-acetylated or N-sulphated and is frequently O-sulphated at C6. The uronic acid moiety can be either glucuronic acid or iduronic acid which is frequently sulphated at C2. Whereas heparin is highly sulphated and contains predominantly iduronic acid, heparan sulphate contains more N-acetyl, less N-sulphate groups and a higher proportion of glucuronic acid. Biosynthetic studies indicate that both polymers can arise from the same precursors by different degrees of polymer modification and explain much of the apparent bewildering complexity of these polysaccharides (McDuffie et al, 1975) (see Lindahl, 1976 for a review). The sequence of polymer modifications occurs as shown in Fig. 3. Some of the reactions take place in a strictly stepwise manner. Other reactions operate in a more concerted, yet sequential, fashion; this applies for instance to the C5-epimerization and 2-O sulphation of uronic acid residues. Due to the substrate specificities of the enzymes involved the individual polymer modification reactions are strongly interdependant. N-deacetylation is thus prerequisite not only to N-sulphation but also to uronosyl C5-epimerization, since only the N-sulphated polymer is recognized as substrate by the epimerase (Lindahl et al, 1977). Polymer sequences that escape N-deacetylation will therefore give rise to low sulphated, D-glucuronic acid-rich regions in the final product. Conversely only N-deacetylated segments may proceed through the series of modification reactions and acquire high contents of sulphate and L-iduronic acid residues. However it is important to note that disaccharide units may remain at various levels of incomplete modification. For example, the N-sulphated disaccharide units identified in heparin and heparan sulphate include not only the extensively modified, di-O-sulphated unit shown as end product, but also the different mono-O-sulphated permutations indicated in Table 2.

1.3.1.5 Keratan Sulphate

Keratan sulphate has many characteristics that distinguish it from other glycosaminoglycans (Fig. 2). It has a repeating disaccharide containing N-acetylglucosamine and galactose but no uronic acid. It is usually of relatively low molecular weight and has been characterized in cornea, cartilage and intervertebral disk.

On the basis of its linkage to protein it has been classified into two types. KS-I occurs in cornea and contains an alkali-stable linkage to protein that involves an N-glycosylamine linkage from

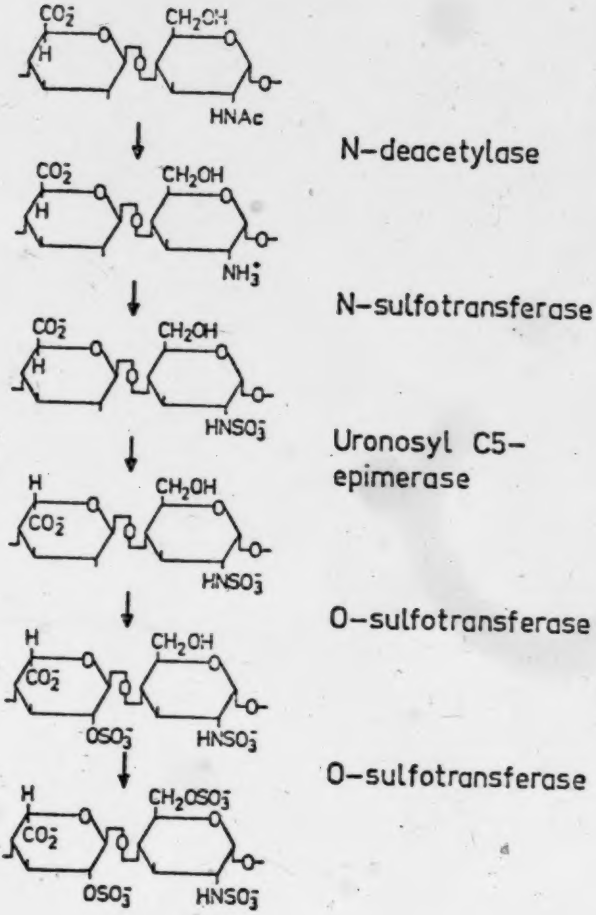

N-deacetylase
N-sulfotransferase
Uronosyl C5-epimerase
O-sulfotransferase
O-sulfotransferase
CO₂⁻
CH₂OH
H
HNAc
NH₃⁺
HNSO₃⁻
OSO₃⁻
CH₂OSO₃⁻

Fig. 3 The Sequence of Polymer Modification Reactions Leading to Formation of the Predominant Disaccharide Unit in Heparin.

N-acetylglucosamine to asparagine or glutamine (Baker et al, 1975). KS-II is found in cartilage and nucleus pulposus and is invariably linked to the same protein as chondroitin sulphate (Tsiganos and Muir, 1967). The available evidence suggests that most of the keratan sulphate chains are attached to the protein through glycosidic bonds between galactosamine and the hydroxyl groups of serine and threonine residues (Bray et al, 1967 and Hopwood and Robinson, 1974). The galactosamine moiety often appears to be substituted on position 3 with a neuraminylgalactosyl disaccharide and on position 6 with the characteristic keratan sulphate chain (Hopwood and Robinson, 1974).

1.3.2. Cartilage Proteoglycans

Proteoglycans are distinguished by the type of glycosaminoglycan chain and by the protein core. By far the most intensively studied proteoglycan is that obtained from hyaline cartilage.

Mathews and Lozaityte (1958) concluded from viscosity and light scattering studies on bovine nasal cartilage proteoglycan that the molecule had a protein core to which a large number of chondroitin sulphate chains were attached so that the total structure took the form of a bottle brush. The molecule had a molecular weight of 4×10^6 and could exist in large aggregate forms. However many of the distinct characteristics of these proteoglycans were not realized until new techniques of extraction and purification were introduced (Sajdera and Hascall, 1969 and Hascall and Sajdera, 1969). Dissociative procedures were used to extract proteoglycans instead of the disruptive homogenization procedures used previously. The proteoglycans were then purified by equilibrium density gradient centrifugation (Franek and Dunstone, 1966). Hascall and Sajdera showed that proteoglycans prepared in this way contained both aggregated and non-aggregated species and that aggregates could be dissociated in 4 M guanidinium chloride. These procedures, which are now in general use in this field, have enabled considerable advances to be made in the understanding of cartilage proteoglycans.

The average proteoglycan molecule from bovine nasal cartilage, for example, has a molecular weight of approximately 2.5×10^6 (Hascall and Sajdera, 1969 and Pasternack et al, 1974). It contains a core protein of about 200,000 molecular weight with about 100 chondroitin sulphate chains, each with an average molecular weight of 20,000 and 30 - 60 keratan sulphate chains, each with molecular weights of 4,000 - 8,000 distributed along special regions of the protein core. (Fig. 4), (Hascall and Riolo, 1972 and Hascall and Heinegård, 1974a). A large proportion of the proteoglycans have a portion of protein, the hyaluronic acid binding region, located at one end of the core (Hardingham and Muir, 1972 and Hascall and Heinegård, 1974a). About 65% of the keratan sulphate chains are localized on another portion of the core, the keratan sulphate enriched region, adjacent to the hyaluronic acid binding region, while more than 90% of the chondroitin sulphate chains are attached to the chondroitin sulphate enriched region, located further from the hyaluronic acid binding region (Fig. 4), (Heinegård and Axelsson, 1977 and Heinegård, 1977). Data from electron-microscopical studies (Thyberg et al, 1975) and proteolytic digestion of the proteoglycan (Heinegård and Hascall, 1974 and Roughley and Barrett, 1977) suggest that the chondroitin sulphate chains are attached to the protein in clusters.

The family of proteoglycan molecules in bovine nasal cartilage is, however, widely polydisperse with molecular weights ranging from a few hundred thousand to more than 4 million (Hascall and Sajdera, 1970). This range of molecular weights is primarily the result of a variation in the number of chondroitin sulphate chains bound to each core protein (Hardingham et al, 1976, Rosenberg et al, 1976 and Heinegård, 1977). It is, moreover, correlated with changes in the overall lengths of proteoglycan cores observed in electron microscopic studies of proteoglycans spread on thin films of cytochrome C (Rosenberg et al, 1975 and Thyberg et al, 1975) and also with changes in the amino acid and hexosamine content. These results suggest that the polypeptide in the chondroitin sulphate enriched region is longer when it contains more chondroitin sulphate chains, whereas the hyaluronic acid binding region remains constant (Hardingham et al, 1976 and Rosenberg et al, 1976 and Heinegård, 1977).

Physicochemical studies show that the glycosaminoglycan chains are extended out from the core and hence the macromolecules occupy large

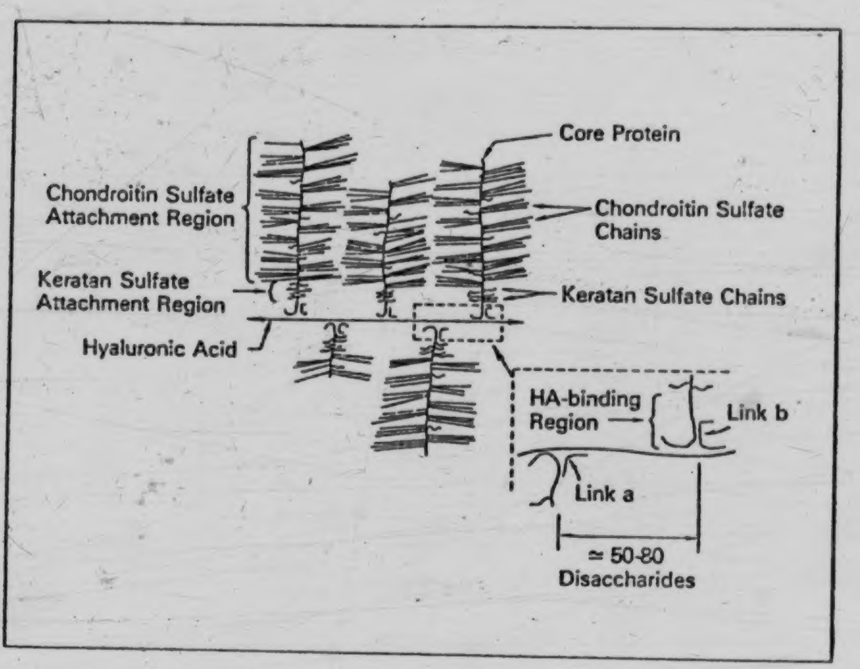

Core Protein
Chondroitin Sulfate Attachment Region
Chondroitin Sulfate Chains
Keratan Sulfate Attachment Region
Keratan Sulfate Chains
Hyaluronic Acid
HA-binding Region
Link b
Link a
≃ 50-80 Disaccharides

Fig. 4 Schematic model for the structure of cartilage protoglycans and the interactions involved in the formation of aggregates. Taken from Hascall (1977).

molecular domains which encompass large amounts of solvent per mass of proteoglycan (Hascall and Sajdera, 1970 and Pasternack *et al*, 1974).

Cartilage proteoglycans generally occur in the tissues as aggregates of very high molecular weight, held together by binding of proteoglycan monomers to hyaluronic acid in the presence of link proteins (Fig. 4), (for review see Muir and Hardingham, 1975 and Hascall, 1977). Individual strands of hyaluronic acid bind as many as 100 proteoglycan monomer molecules (Hardingham and Muir, 1972 and 1973 and Hascall and Heinegård 1974a and b) to form aggregates. Although the interaction is not covalent, it is extremely specific to hyaluronic acid and does not occur with any other glycosaminoglycan even those as closely related as chondroitin (desulphated chondroitin sulphate; Hascall and Heinegård, 1974b) which differs from hyaluronate only in the conformation of the hydroxyl group on C-4 of the hexosamine residues or an intermediate in heparin biosynthesis, $[(1\text{-}4)\beta\text{-D-glucuronosyl-}\ (1\text{-}4)\alpha\text{-D-N acetylglucosaminyl}]_n$ which differs from hyaluronate only in the glycosidic linkage (D. Heinegård, personal communication). The minimum length of hyaluronate that binds strongly is nine sugar residues. Oligosaccharides of hyaluronate, containing 9 or more sugar residues, compete strongly with hyaluronic acid and inhibit the interaction whereas octasaccharides and smaller oligosaccharides have little effect (Hardingham and Muir, 1973, Hascall and Heinegård, 1974b and J. Christner, personal communication). The hyaluronate-binding region, however, is quite large. Core protein fragments with molecular weights in the order of 60 - 70,000, which contain the hyaluronate-binding region, could be isolated after mild proteolytic digestion of the aggregates (Hascall and Heinegård, 1974a, Heinegård and Hascall, 1974 and Oegema *et al*, 1975).

Proteoglycan aggregation is prevented by reduction of cystine residues (Hascall and Sajdera, 1969), suggesting that the conformation of the hyaluronate-binding region is critical for the binding process. Selective modification of amino acid residues in the core protein indicated that binding to hyaluronate also required intact arginine and tryptophan residues as well as ϵ-amino groups of lysine (Hardingham *et al*, 1976).

Hyaluronate-proteoglycan interaction can be demonstrated in the absence of additional components (Hardingham and Muir, 1973, Hascall and Heinegård, 1974a and Swann et al, 1976), but it is stabilized by two link proteins that form part of the native aggregates (see Hascall, 1977 for a review). The link proteins are structurally closely related (Baker and Caterson, 1977) and are able to bind hyaluronate in the absence of proteoglycan and proteoglycan in the absence of hyaluronate (Caterson and Baker, 1978).

Besides proteoglycans that interact with hyaluronate, there are some that do not do so. These are not present as aggregates and may be preferentially extracted with 0.15 M NaCl. They contain less cystein and tryptophan and have a small size and low protein and keratan sulphate content (Hardingham and Muir, 1974 and Mayes et al, 1973). It has been suggested that these molecules may be fragments, containing the chondroitin sulphate-bearing region of aggregating proteoglycan, produced by proteolytic cleavage (Hardingham et al, 1976). The addition of a mixture of proteinase inhibitors to 0.15 M KCl extracts of bovine nasal septum cartilage increased the proportion of aggregating proteoglycan from 10 to 42% of the proteoglycan extracted (Pearson and Mason, 1977). However this extract still contained a large proportion of non-aggregating proteoglycan (58% of the total extracted), which may be a distinct species of proteoglycan or a proteoglycan breakdown product formed prior to extraction.

1.3.3 Proteoglycans of Tissues other than Cartilage

There is much less information about proteoglycans in tissues other than cartilage. This stems from the lower amounts of proteoglycan found in these tissues and the consequent problems involved in purifying small amounts of proteoglycan in the presence of large quantities of non-proteoglycan protein, particularly collagen. Another problem that deterred these studies was the early observation that the dermatan sulphate proteoglycans were much more difficult to extract by the techniques in vogue at the time. Lowther and co-workers found that whereas chondroitin sulphate proteoglycans and hyaluronic acid could be extracted by homogenization in water (Meyer et al, 1969) or 0.2 M NaCl (Lowther et al, 1970) the dermatan sulphate proteoglycans could only be extracted, in reasonable yield, with 6M urea at 60°C.

Chondroitin sulphate proteoglycans extracted from bovine heart valves (Lowther et al, 1970) and bovine aorta (Kresse et al, 1971, Antonopoulos et al, 1974, Ehrlich et al, 1975 and Radhakrishnamurthy et al, 1977) differ from those in cartilage in being of lower molecular weight, in lacking keratan sulphate and in containing small amounts of dermatan sulphate (at least in the case of the aorta proteoglycans). There appears to be a number of chondroitin sulphate proteoglycan species in heart valves. Lowther et al (1970) found that those proteoglycans extracted with 0.2 M NaCl were smaller (molecular weight approximately 40,000 c.f. 65,000), contained less protein (5% c.f. 30%), and had a higher chondroitin 6-sulphate: chondroitin 4-sulphate ratio (1.5 c.f. 0.6) than those extracted with 1 M NaCl. Similar proteoglycans have been extracted from bovine aorta with 3.0 M $MgCl_2$ (Ehrlich et al, 1975) or by digestion with collagenase (Radhakrishnamurthy et al, 1977), although these appeared slightly larger (molecular weight 72,000) and contained a small amount of dermatan sulphate (7% of the total glycosaminoglycan). Although no dermatan sulphate was reported in the heart valve proteoglycan preparations, the analyses performed suggested that low levels of this order may be present.

Chondroitin sulphate proteoglycans approaching the size of those extracted from cartilage have been extracted from aorta with 0.15 M phosphate buffer(Kresse et al, 1971) or with 4 M guanidinium chloride (Antonopoulos et al, 1974). These proteoglycans had a chemical composition similar to the smaller chondroitin sulphate proteoglycans. From hyaluronidase digestion studies Kresse et al (1971) concluded that the dermatan sulphate and chondroitin sulphate were linked to the same protein core and that about 50% of the dermatan sulphate units occurred as dermatan sulphate-chondroitin sulphate copolymers.

The similarity in glycosaminoglycan composition and broad range of molecular sizes observed with these proteoglycans, may reflect the action of endogenous proteases either active in the tissue or in the tissue extracts. This possibility was recognized by all the authors cited who took measures either to minimize the activity or detect its presence. However, although no endogenous protease activity was detected, the possible occurrence of a small number of proteolytic cleavages, sufficient to reduce the molecular weight of the molecules from approximately 2 million (cartilage proteoglycan subunit) to 50,000 - 100,000 could not be eliminated.

A dermatan sulphate proteoglycan was extracted with hot concentrated urea from bovine heart valves (Toole and Lowther, 1968b). It was shown to contain about 50% protein and was polydisperse with a molecular weight of $1 - 2 \times 10^5$ (Preston, 1968). Dermatan sulphate proteoglycans with very similar protein content (45 - 58%) and amino acid composition have been obtained from bovine achilles tendon by 3.0 M $MgCl_2$ extraction (Anderson, 1975) and from bovine skin (Öbrink, 1972) by hot urea extraction. The molecular weight of the tendon proteoglycan seemed similar to that isolated from heart valves, however the molecular weight of the skin preparation was determined to be 2.0×10^6 using light scattering. This higher estimate could arise from the presence of large aggregates which have been shown to form with this material (Toole and Lowther, 1968b).

Axelsson and Heinegård have recently reported the isolation (Axelsson and Heinegård,1975) and careful chemical and physical characterization (Axelsson and Heinegård, 1978) of keratan sulphate proteoglycans that accounted for approximately one-third of the proteoglycans and a large proportion of the keratan sulphate of the bovine corneal stroma. The proteoglycans contain monomers with molecular weights of about 72,000, in addition to slightly larger polydisperse molecules, with the same chemical composition of about 45% protein, 30% keratan sulphate (the only glycosaminoglycan present) and 10 - 12% oligosaccharides. The molecules appeared to contain intrachain disulphide bonds, one polypeptide chain, one to three keratan sulphate chains and approximately 12 oligosaccharide chains per molecule. The proteoglycans were able to form aggregates and aggregation was promoted by the large molecular size fraction of the keratan sulphate proteoglycans, by low pH and reduction and alkylation of disulphide bonds. Non-proteoglycan factors promoting aggregation could not be detected.

Thus there are a number of proteoglycan species that are unique in their physical characteristics and in their glycosaminoglycan and protein composition. The proteoglycans of non-cartilaginous tissues tend to be smaller and contain more protein than their cartilaginous counterparts, though the molecular sizes of the proteoglycans obtained should be viewed with some reservation due to the possibilities of proteolytic cleavage prior to or during extraction and purification (see Oegoma et al, 1975). There is some indication that proteoglycans with similar glycosaminoglycan composition may have homologous structures.

1.4 THE PHYSIOLOGICAL FUNCTIONS OF PROTEOGLYCANS

Most of the understanding we have of the physiological function of proteoglycans, small though it is, has come from the investigation of the physical properties of cartilage proteoglycans (see Comper and Laurent, 1978 and Lindahl and Hook, 1978 for comprehensive reviews of glycosaminoglycan and proteoglycan function).

Cartilage proteoglycans are extremely large molecules that have a highly expanded structure in solution. Because of these properties and their ability to aggregate with hyaluronic acid, they become entangled and immobilized in the collagen network of the cartilage. They impede the flow of interstitial water when an external force is applied, thus giving the tissue elasticity and resilience to compressive forces. The hydrophilic nature of proteoglycans and collagen induces a swelling pressure in the tissue to which osmotic pressure also contributes. Thus proteoglycans are immobilized polyanions and therefore mobile counter ions become more concentrated in their vicinity creating a Donnan equilibrium and a large positive osmotic pressure within the tissue. The swelling pressure of connective tissue thus increases with the proteoglycan content (Comper and Laurent, 1978).

Proteoglycans also act as molecular sieves as a consequence of their steric exclusion of other solutes. This helps to explain the striking effect of 'spreading factor' (hyaluronidase) on the flow of water in subcutaneous tissue (Chain and Duthie, 1940) and the almost complete exclusion of molecules the size of immunoglobulins from the cartilage matrix (Poole et al, 1973).

Proteoglycans as a consequence of their branched polyanionic character and their entanglement and immobilization in tissues have been viewed as a phase similar to an anion exchange resin and many of their properties have been interpreted in this fashion. They are capable of immobilizing large quantities of counterion: their interaction with which appears to depend on the linear charge density of the glycosaminoglycan chains and the valence of the counterion (Comper and Laurent, 1978). The interaction of calcium ions with proteoglycans has received considerable attention in view of the obvious role of calcium in tissue mineralization. Though proteoglycans do appear to bind significant amounts of Ca^{2+} (Farber et al, 1957, MacGregor and Bowness, 1971 and Smith and Lindenbaum, 1971) no marked differences have been observed in the behaviour of free glycosaminoglycan chains, proteoglycan monomers and proteoglycan aggregates (Cuevero et al, 1973). Several lines of evidence favor a relatively high affinity for calcium for proteoglycan fractions associated with collagen (Smith and Lindenbaum, 1971). It has been reported (Cuevero et al, 1973 and Smith and Lindenbaum, 1971) that aggregate forms of proteoglycans possess a potent inhibitory effect on mineral growth in vitro in comparison with non-aggregate forms. These observations are consistent with the observed decrease in size and aggregation of the proteoglycans of the epiphyseal plate (Howell and Pita, 1976) and matrix induced cartilage (Reddi et al, 1978) with increased calcification and subsequent ossification.

Although few detailed structure-function relationships have been postulated, the functions so far described would not seem to warrant the structural complexity and variability observed with glycosaminoglycans and proteoglycans. It seems likely that other functions of proteoglycans may involve their binding to biological macromolecules. Diverse patterns of binding have been demonstrated, including not only polysaccharide-protein but also polysaccharide-polysaccharide interactions. Some of these binding reactions have been implicated in such phenomena as the formation of atherosclerotic plaque and the control of hemostasis.

Fransson (1976) recently reported the binding of copolymeric dermatan sulphate - chondroitin sulphate chains as well as homopolymeric glycosaminoglycans to dermatan sulphate-substituted agarose gels. The most pronounced interaction occurred if the copolymeric chains contained similar proportions of L-iduronic acid and D-glucuronic acid. However chondroitin 4-sulphate, heparan sulphate and heparin also showed interaction with gels substituted with copolymeric glycosaminoglycans, while chondroitin 6-sulphate, hyaluronate and keratan sulphate chains did not. No physiological role for this interchain interaction has, as yet, been found, however selective polysaccharide chain aggregation under physiological conditions offers prospects for future research on the functional role of copolymeric glycosaminoglycans in the intercellular space and, particularly, on the cell surface (see Malmström et al, 1975).

One type of glycosaminoglycan or proteoglycan interaction which has been dealt with in considerable detail due to its obvious physiological significance, is the binding between polysaccharides and collagen. This interaction has been demonstrated by a number of techniques (see Öbrink, 1975 for a review) including electrophoresis (Mathews, 1965), affinity chromatography (Öbrink and Wasteson, 1971 and Greenwald et al, 1975), precipitation (Toole and Lowther, 1968 a and b), light scattering (Öbrink and Sundelof, 1973), agglutination of collagen-coated erythrocytes (Conochie et al, 1975) and circular dichroism spectroscopy (Gelman and Blackwell, 1974). The results indicate that the binding is electrostatic, that glycosaminoglycans of higher charge density and large molecular size bind with higher affinity and that the presence of L-iduronic acid residues appears to promote the binding process. Proteoglycans interact more strongly than the individual chains and the protein core seems capable of interacting in the absence of the glycosaminoglycan chains (Oegema et al, 1975 and Greenwald et al, 1975).

It has been implied that the interaction between proteoglycans and collagen could be important during collagen fibre formation. Most studies have examined the kinetics of fibre formation turbidimetrically by refinements (Wood and Keech, 1960) of a technique described by Gross and Kirk (1958). The effects of proteoglycans on collagen fibre formation however, are difficult to interpret due to apparently conflicting results that arise largely from differences in the homogeneity of the collagen preparations (Öbrink, 1973). However Oegema et al, 1975, using a monomeric collagen preparation recently concluded that proteoglycans have two distinct effects on collagen fibrillogenesis: they retard the assembly of collagen if present early enough during the process, and they alter the final organization of the fibril as visualized through electron microscopy. Furthermore the proteoglycan molecules were tightly bound to the collagen fibrils only if present before fibril formation began. Individual chondroitin sulphate chains, large trypsin fragments with one to eight chondroitin sulphate chains per peptide and the slightly larger cathepsin D fragments had no effect on fibril formation. Isolated core protein molecules while capable of binding to the forming fibrils, did not alter the kinetics of fibre formation.

The interaction of glycosaminoglycans with lipoproteins has been investigated by a variety of techniques including precipitation (Srinivasan _et al_, 1975 and Nakashima _et al_, 1975) and equilibrium binding of lipoproteins to polysaccharide-substituted agarose gels (Iverius, 1972 and Srinivasan _et al_, 1975). Low density and very low density lipoproteins were found to bind glycosaminoglycans. Binding depended on electrostatic forces, increased with charge density of the polysaccharide and as with many other interactions preferential binding of species containing L-iduronic acid was noted. Heparin and other iduronic acid containing glycosaminoglycans also bind to lipoprotein lipase (Olivecrona _et al_, 1977). The presence of this type of glycosaminoglycan in the blood vessel wall may conceivably provide a means for selective binding of plasma components and perhaps a mechanism for the formation of atherosclerotic plaques.

Several of the proteins of the blood coagulation system also bind glycosaminoglycans. Heparin appears to bind specifically to antithrombin (Li _et al_, 1976, Hopwood _et al_, 1976 and Einarsson and Andersson, 1977) thereby facilitating thrombin antithrombin interaction and hence inhibition of thrombolytic activity. Heparan sulphate and dermatan sulphate are also able to prevent blood coagulation, though at higher concentrations and by unknown mechanisms (Teien _et al_, 1976). Platelet factor 4, which mediates the anticoagulant effect of heparin, is displaced from its natural proteoglycan carrier by heparin as well as heparan sulphate and dermatan sulphate (Olivecrona _et al_, 1977) further implicating these glycosaminoglycans in the control of hemostasis.

Glycosaminoglycans have also been implicated in a large number of other binding reactions. Nuclei from different kinds of cells appear to contain significant amounts of glycosaminoglycans (Kinoshita, 1974, Bhavanandan and Davidson, 1975, Stein et al, 1975 and Fromme et al, 1976) and these have been suggested to affect translation (Waldman et al, 1974 and Goldstein et al, 1975) as well as transcription (Arnold et al, 1972, Cook and Aikawa, 1973 and Schaffrath et al, 1976) of genetic information. Glycosaminoglycans (hyaluronic acid, chondroitin sulphates and heparan sulphate) have been found in neural tissues and implicated in the processes of neurotransmission and the storage and release of biogenic amines and hormones (see Margolis and Margolis, 1977 for a review). Sulphated glycosaminoglycans have also been identified as constituents of various storage and secretory granules (Lagunoff et al, 1964, Schuel et al, 1974, Blaschke et al, 1976, Giannattasio and Zanini, 1976 and Margolis and Margolis, 1973). The functions of the glycosaminoglycan in these granules may be similar to that proposed for heparin in the mast cell granules where heparin occurs as an insoluble complex with a basic small protein and this complex acts as a store for histamine (Bergqvist et al, 1971). Titration data suggested that the histamine molecules are primarily bound to carboxyl groups in the protein; the protein in turn is electrostatically bound to sulphate groups in the polysaccharide. During degranulation and subsequent exposure of the heparin - protein - histamine complex to the cations (mainly Na^+) of the extracellular fluid, histamine is liberated by a simple cation-exchange mechanism (Uvnas, 1974 and Uvnas and Åborg, 1976).

Thus a large number of macromolecules may bind to glycosaminoglycans. The majority of ligands are proteins or protein conjugates, though some glycosaminoglycans appear capable of interchain binding. The general interactions of glycosaminoglycans are facilitated by increased charge density and by stereochemical factors that are not understood but which arise from the presence of L-iduronic acid residues. Other than the specific interaction of cartilage proteoglycan with hyaluronic acid (see section 1.3.2) the involvement of the respective proteoglycans in binding reactions has received little attention.

1.5 AIMS OF THE PRESENT INVESTIGATION

The proteoglycan components of the periodontal ligament have been implicated in many of its functions, in particular in the process of tooth support and tooth movement. Yet the structure and composition of these proteoglycans is largely unknown despite the fact that periodontal disease, which results in destruction of this tissue, is widespread and affects about 80% of adults (Freedman and Grainger, 1965).

The present investigation was therefore initiated in order to gain some insight into the detailed structure of the ligament glycosaminoglycans, into their changes associated with ligament development and hence some idea of their association with tooth eruption. From the study of their proteoglycan structure we hoped to achieve a closer understanding of their possible functions and in association with the study of proteoglycans from the skin and cartilage we hoped to expand our understanding of the structure of non-cartilaginous proteoglycans in general.

CHAPTER 2

MATERIALS and METHODS

2.1 MATERIALS

Papain, 2 x crystallized and hyaluronidase, type V from ovine testes were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Chondroitin ABC lyase and the standard glycosaminoglycans (Chondroitin sulphate type A, chondroitin sulphate type B, chondroitin sulphate type C, and hyaluronic acid) were obtained from Miles Research Products, Elkhart, Indiana, U.S.A. Cathepsin D was isolated from bovine thymus and purified by Dr. Pearson (Scott and Pearson, 1978). The stock solution contained 100 μg/ml. Cathepsin B obtained from human liver and human leucocytic elastase were gifts from Dr. A. J. Barrett. Aqueous stock solutions contained 1.9 mg/ml (specific activity 4.5 units/mg, Starkey and Barrett, 1973), and 1.8 mg/ml (specific activity 72 units/mg, Starkey and Barrett, 1976) respectively.

Gel chromatography media (Sephadex G-25, G-50, and G-200, Sepharose 2B, 4B and 6B) were obtained from Pharmacia (Canada) Ltd., Dorval, Quebec. DEAE-cellulose (DE-52) was obtained from Whatman Biochemicals, Ltd., Maidstone, Kent, England.

Chemicals used for composite gel electrophoresis were of electrophoresis grade and obtained from BioRad Laboratories, Richmond, California, U.S.A.

The urea solutions used in this work were purified by passage through a mixed bed ion-exchange resin of the type commonly used to deionize water.

Guanidinium chloride, used in most experiments, was ultra pure grade obtained from Schwarz/Mann, Orangeburg, N.Y., U.S.A. When solutions of a lower grade of guanidinium chloride were used they were first purified by stirring with activated charcoal (Norit) and then filtered.

Bovine mandibles from cattle aged 1 - 2 years were obtained fresh from the abattoirs. After fracturing the bones, unerupted, partially erupted and mature occluded incisors were easily removed. These were then sorted into six groups, each group representing a different stage of tooth development. The criteria used to classify the teeth were size, weight, root formation, position in the jaw and attrition.

Group	Wet Weight gm	Root length mm	Intraoral	Occluded	Ligament
1	0.6 -2.5	0	-	-	-
2	2.69-3.75	2-5	-	-	+
3	3.60-5.65	6-18	-	-	+
4	5.50-7.90	19-27	-	-	+
5	5.50-9.20	19-27	+	-	+
6	6.0 -10.0	19-27	+	+	+

Table 4. Bovine Incisor Classification

Root length was measured from the cemento-enamel junction to the root apex. Group 1 were small unerupted teeth showing no sign of root formation. Group 2 were small unerupted teeth showing the first signs of root formation. Group 3 were unerupted teeth showing prominent root formation. Group 4 were also unerupted teeth showing prominent root formation, however the crowns of these teeth were in contact with the oral mucosa and appeared to be close to eruption into the oral cavity. The teeth from group 5 had just broken the oral mucosa and those in group 6 were mature occluded teeth.

The ligament was removed from the cement surface of the root with gentle scraping, care being taken to exclude connective tissue surrounding the root apex and the small amounts of alveolar bone adhering to the ligament. Ligaments used in the isolation of proteoglycans, because of the limited material available, were pooled from teeth in groups 3, 4, 5 and 6. Ligaments were cut into small pieces, frozen in liquid nitrogen and milled using a Wiley Mill cooled with liquid nitrogen. Care was taken to keep the incisors and ligaments chilled on ice at all times.

Bovine nasal septa were obtained from a local abattoir, chilled on ice and brought to the laboratory within 1-2 hrs. after slaughter. The perichondrium was removed and the cartilage cut into small pieces with a scalpel, frozen in liquid nitrogen and milled in a Wiley Mill cooled with liquid nitrogen.

Bovine skin was obtained from a 1 to 2 year old steer, freed of hair and subcutaneous tissue and cut into small pieces. This material was frozen in liquid nitrogen and milled in a Wiley Mill cooled with liquid nitrogen.

2.2 METHODS

2.2.1 Isolation and Fractionation of Glycosaminoglycans

2.2.1.1 Extraction

Samples of periodontal ligament (4 to 10 gm wet weight) were dried to constant weight at 110° C then defatted with several changes of acetone. This material was digested with papain (1 mg papain/50 mg dry weight tissue) in papain buffer at 65° C for 16 hrs. The buffer contained 0.1 M sodium acetate, 5 mM EDTA, pH 6.0, 1 mg/ml cysteine and a few drops of toluene as bacteriostatic agent. After 16 hrs. an additional 0.5 mg papain/ 50 mg dry weight was added and the incubation continued a further 24 hrs. After papain digestion 100% (w/v) trichloracetic acid (TCA) was added to a concentration of 6% and left at 4° C for 30 min. to precipitate nucleic acid and partially digested protein. The residue was collected by centrifugation. In order to decrease the amount of residue after papain digestion some samples were made to 0.5 M in NaOH prior to centrifugation and kept at 4° C overnight. This treatment made no difference to the total uronic acid analyses and suggested that little or no uronic acid was present in the residue after papain digestion.

2.2.1.2 CPC Precipitation of Glycosaminoglycans

A flow diagram for the fractionation procedure is given in Fig. 5. The supernatants after TCA precipitation were dialysed against several changes of distilled water for 24 - 48 hrs., at room temperature, and

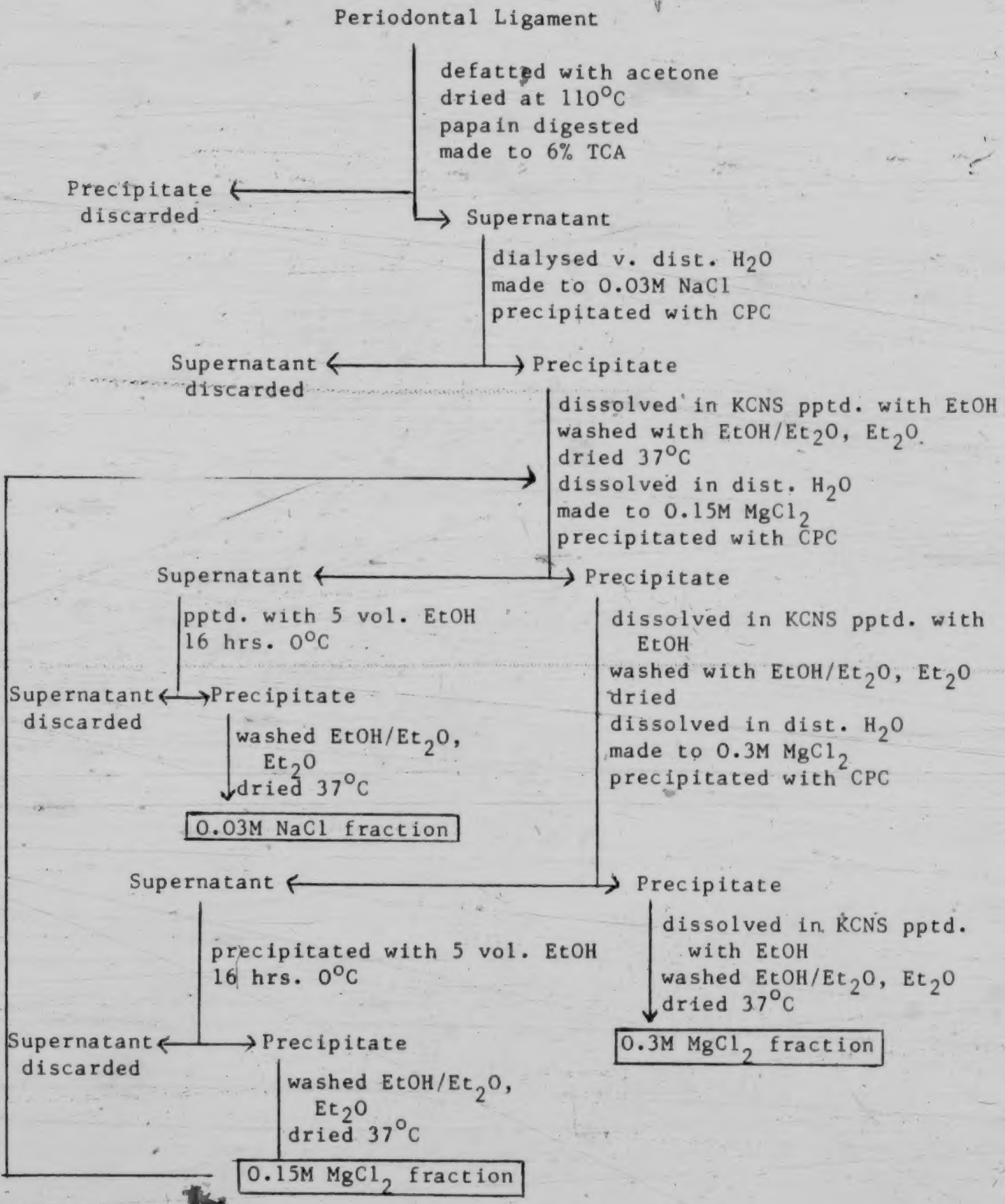

Periodontal Ligament
defatted with acetone
dried at 110°C
papain digested
made to 6% TCA
Precipitate discarded
Supernatant
dialysed v. dist. H2O
made to 0.03M NaCl
precipitated with CPC
Supernatant discarded
Precipitate
dissolved in KCNS pptd. with EtOH
washed with EtOH/Et2O, Et2O
dried 37°C
dissolved in dist. H2O
made to 0.15M MgCl2
precipitated with CPC
Supernatant
Precipitate
pptd. with 5 vol. EtOH
16 hrs. 0°C
Supernatant discarded
Precipitate
washed EtOH/Et2O, Et2O
dried 37°C
0.03M NaCl fraction
dissolved in KCNS pptd. with EtOH
washed with EtOH/Et2O, Et2O
dried
dissolved in dist. H2O
made to 0.3M MgCl2
precipitated with CPC
Supernatant
Precipitate
precipitated with 5 vol. EtOH
16 hrs. 0°C
dissolved in KCNS pptd. with EtOH
washed EtOH/Et2O, Et2O
dried 37°C
0.3M MgCl2 fraction
Supernatant discarded
Precipitate
washed EtOH/Et2O, Et2O
dried 37°C
0.15M MgCl2 fraction

Fig. 5 CPC Precipitation of Ligament Glycosaminoglycans

EtOH - ethanol

Et_2O - diethyl ether

then made to 0.03 M in NaCl. Glycosaminoglycans were precipitated by the dropwise addition of 5% cetyl pyridinium chloride (CPC) at 37° C until no further precipitation occurred. After 60 min. at 37° C, the CPC precipitate was pelleted on a bench centrifuge, then dissolved in 2.0 M KCNS in 20% (v/v) ethanol to exchange potassium for the cetyl pyridinium ions on the glycosaminoglycans. The glycosaminoglycans were then reprecipitated by the addition of 5 volumes of ethanol and incubated at 0° C for 16 hrs. The precipitates were then centrifuged, washed with ethanol/ether (1:1), then ether, and dried at 37° C. These precipitates were then dissolved in distilled water, made to 0.15 M with respect to $MgCl_2$, precipitated with CPC and pelleted. In contrast to the CPC precipitates in 0.03 M NaCl, these precipitates (and those in 0.3 M $MgCl_2$) often required higher speed centrifugation (15,000 g for 15 mins.) before they formed a pellet. The precipitates were dissolved in 2.0 M KCNS in 20% ethanol, reprecipitated with ethanol, washed with ethanol/ether, then ether and dried at 37° C as described above. 5 volumes of ethanol were added to the CPC supernatant. This was kept at 0° C for 16 hrs., pelleted, washed with ethanol/ether and dried at 37° C. This fraction was termed the 0.03 M NaCl fraction. The glycosaminoglycans precipitated from the 0.15 M $MgCl_2$ solution were dissolved in water, made to 0.3 M in $MgCl_2$ and precipitated with 5% CPC. The precipitate was pelleted, dissolved in 2.0 M KCNS in 20% ethanol, reprecipitated with ethanol, washed with ethanol/ether and then ether and dried at 37° C as described above. This fraction was termed the 0.3 M $MgCl_2$ fraction. The glycosaminoglycans in the supernatant were precipitated by the addition of 5 volumes of 98% ethanol as described above and washed with ethanol/ether, then ether and dried at 37° C as described above. Since this fraction appeared, by cellulose acetate electrophoresis, to be contaminated with hyaluronic acid and chondroitin sulphate, it was made to 0.15 M $MgCl_2$ and refractionated as shown in the flow chart.

2.2.1.3 Ethanol Precipitation of Glycosaminoglycans

The 0.3 M $MgCl_2$ fractions were dissolved in water and made to 5% calcium acetate, 0.5 M acetic acid by the addition of an equal volume of 10% calcium acetate, 1.0 M acetic acid. This solution was fractionated with ethanol essentially as described by Meyer et al, (1956). Ethanol was added dropwise with vigorous stirring and each fraction was left for at least 24 hours at 0° C. The precipitates were centrifuged, then washed twice with 80% (v/v) ethanol, then ethanol/ether, then ether and dried at 37° C.

Four fractions were collected at 18, 25, 40 and 50 percent ethanol. In initial experiments a further fraction was precipitated at 30 percent ethanol. This contained a very small fraction of the total sulphated glycosaminoglycans and appeared, by cellulose acetate electrophoresis, to be a mixture of the 25 and 40 percent ethanol fractions. In some experiments the 40 percent ethanol fraction was refractionated at ethanol concentrations of 30, 40 and 50 percent.

2.2.2 Enzymic and Chemical Digestion of the Glycosaminoglycans

2.2.2.1 Hyaluronidase Digestion

The glycosaminoglycan samples were digested with ovine testicular hyaluronidase (Sigma type V) (1432 NF units/mg glycosaminoglycan) in 0.1 M sodium acetate, 0.1 M NaCl, pH 5.0 at 37° C for 18 hours. The reaction was stopped by 2 mins. incubation in a boiling water bath, the NaCl concentration was reduced to 0.03 molar and the glycosaminoglycans remaining were precipitated with 5 percent CPC as described previously (see section 2.2.1.2). Since hyaluronidase cleaves only β-hexosaminic bonds to D-glucuronic acid and not to L-iduronic acid, the uronic acid content of the precipitated material was used as a determination of the dermatan sulphate content. However, as only 80 percent of standard dermatan sulphate was recovered in the precipitate by use of this technique, calculations of the dermatan sulphate content of glycosaminoglycan samples were corrected for this digestion. That is, the uronic acid content of the precipitate was multiplied by 1.25 to obtain the uronic acid in dermatan sulphate. The chondroitin sulphate content was determined by difference or by analysis of the supernatant.

The nature of the digested material, prior to CPC precipitation, was also examined by gel filtration on Sephadex G-50 and Sephadex G-200. The columns were eluted with pyridine acetic acid buffer (0.2 M in acetic acid), pH 5.0 (see section 2.2.4).

2.2.2.2 Chondroitin ABC Lyase Digestion

Samples of glycosaminoglycan were digested with chondroitin ABC lyase essentially as described by Saito et al, (1968). Chondroitin ABC lyase was stored in 50 mM Tris-HCl buffer, pH 8.0 at 0° C and released 0.01 µmol of hexuronic acid per min. per µl of enzyme solution at 37° C from standard chondroitin sulphate. Distilled water solutions of the appropriate glycosaminoglycan fractions, containing approximately 150 µg as uronic acid, were lyophilized and dissolved in 50 µl of 0.1 M Tris-HCl, pH 8.0. Chondroitin ABC lyase (10 µl) was added and the solution incubated at 37° C for 16 hrs. The products were spotted on Whatman 3 MM paper and subjected to descending chromatography for 40 hrs. in isobutyric acid - 2 M NH_4OH (5:3) v/v. After the papers were dried thoroughly the unsaturated disaccharides could be visualized under ultra violet light (the products of chondroitinase digestion could not be seen under ultraviolet light unless the papers were thoroughly dried in a drying oven). The papers were cut into strips and the material eluted with distilled water at 100° C and analysed by uronic acid determination or absorbance at 232nm.

Standard samples of chondroitin 4-sulphate, chondroitin 6-sulphate and dermatan sulphate, digested as described, were subjected to descending chromatography and the mobility of the resultant disaccharides served as references for the digested ligament glycosaminoglycans.

2.2.2.3 Periodate Oxidation and Alkaline Degradation

Glycosaminoglycan samples suspected of being copolymeric were oxidized (Fransson and Carlstedt, 1974) with 20 mM sodium metaperiodate, adjusted to pH 2.0 with 0.1 M HCl, in the dark, at 4° C for 24 hrs. (2 mg. glycosaminoglycan per ml of periodate solution). The reaction was terminated by the addition of 0.1 volumes of 10% aqueous D-mannitol. Oxidized iduronate residues were then cleaved by adjusting the pH to 12 with 0.5 M NaOH and incubating 30 mins. at room temperature. In later experiments samples were dialysed extensively against distilled water before alkali cleavage. This made no difference to the behaviour on gel chromatography but eliminated salt interference near the column total volume. The pH was then adjusted to 5.0 with 1 M acetic acid and the oxidized alkali cleaved samples were subjected to gel chromatography on Sephadex G-200 or Sephadex G-50 eluting with 0.2 M pyridine acetate buffer at pH 5.0.

The effect of pH on the oxidation of standard glycosaminoglycan samples was examined by adjusting the pH of the sodium metaperiodate solution to 2.5 and 3.0. In some cases 50 mM citrate buffer was included in the sodium metaperiodate solutions, but had no discernible effect on the oxidation or subsequent cleavage.

2.2.2.4 Digestion with Nitrous Acid

Glycosaminoglycan fractions suspected of containing heparan sulphate (0.15 M NaCl fractions from CPC precipitation) were digested in nitrous acid, 50 µl each of 33% acetic acid and 5% $NaNO_2$ was added to a 50 µl solution of glycosaminoglycan (1 mg/ml) (Lagunoff and Warren, 1962). After incubation for 2 hrs. at room temperature the mixture was frozen, lyophilized and dissolved in 50 µl of distilled water for cellulose acetate chromatography.

2.2.3 Molecular Weight Determination of Glycosaminoglycans by End Group Estimation Using Tritiated Sodium Borohydride Reduction

2.2.3.1 Alkaline Cleavage and Borohydride Reduction

Glycosaminoglycan fractions were prepared from periodontal ligament by papain digestion, CPC precipitation and alcohol fractionation as described previously. Proteoglycans were prepared from bovine nasal septa, skin and periodontal ligament as described in section 2.2.6 . They were purified by DEAE-cellulose chromatography (except cartilage proteoglycan), cesium chloride density gradient centrifugation and gel chromatography on Sepharose 2B or Sepharose 6B.

The carbohydrate-protein (or peptide in the case of the papain isolated glycosaminoglycans) linkages between xylose and the serine hydroxyl groups were cleaved with dilute alkali and the exposed xylose residues reduced and radioactively labelled with tritiated borohydride as described by Robinson and Hopwood (1973).

Freeze dried proteoglycans or glycosaminoglycans (approximately 250 µg as uronic acid) were gently dissolved in 1 ml of 0.02 M NaB^3H_4 in 0.5 KOH and stirred at 4° C for 10 days. A slight turbidity was noticed in the ligament and skin proteoglycans after this time and it was removed by centrifugation at 15,000 g and 4° C for 30 min. Excess borohydride was destroyed by acidification to pH 5.0 with 2 M acetic acid and the reaction mixtures desalted on a Sephadex G-25 column (1.5 x 25 cm) equilibrated with pyridine-acetic acid buffer pH 5.0 (0.2 M in acetic acid). The column was eluted with the same buffer at a flow rate of 25 ml per hour and 2 ml fractions were collected and assayed for uronic acid content and radioactivity. All the hexuronate positive material was eluted close to the void volume of the column and these fractions were combined and lyophilized.

These glycosaminoglycans were then dissolved in 0.03 M NaCl, precipitated with CPC, and then fractionated by CPC and alcohol precipitation in a manner identical to the isolation of ligament glycosaminoglycans, as described in sections 2.2.1.2 and 2.2.1.3 . The fractions were then dissolved in water and analysed for uronic acid and radioactivity.

The end-labelled glycosaminoglycans, obtained from the proteoglycans and the ligament glycosaminoglycan preparations were applied to a column of Sephadex G-200, prepared and eluted as described in section 2.2.4. 2 ml fractions were collected and analysed for uronic acid and radioactivity.

In order to determine molecular weights a knowledge of the uronic acid content of the glycosaminoglycans and the specific activity of NaB^3H_4 was required.

2.2.3.2 Uronic Acid Content of Glycosaminoglycan Samples

Glycosaminoglycan fractions obtained from the periodontal ligament as described in Fig. 5 were reprecipitated from 5% calcium acetate, 0.5 M acetic acid solutions with two volumes of ethanol and washed thoroughly, as described in section 2.2.1.3. The potassium salt of chondroitin sulphate was obtained from cartilage proteoglycan by papain digestion and precipitation with CPC from solutions 0.3 M in $MgCl_2$ as described for ligament glycosaminoglycans in section 2.2.1.2 . The glycosaminoglycan samples were dried thoroughly in a vacuum oven, over P_2O_5 at 60° C. Dried samples were weighed on an electronic microbalance, dissolved in distilled water and analysed for uronic acid. The following analyses were obtained.

Alcohol fraction	μg uronic acid/mg dry wt.
18	304
25	308
40	339
50	350
chondroitin sulphate	330

Table 5. The Uronic Acid Content of Galactosaminoglycan Samples

The uronic acid contents of the skin glycosaminoglycans were not determined due to lack of material but were assumed to be the same as the ligament glycosaminoglycans.

2.2.3.3 Standardization of Tritiated Sodium Borohydride

Tritiated sodium borohydride was standardized by the end labelling of a sample of chondroitin sulphate of known molecular weight. Chondroitin sulphate was isolated from bovine nasal cartilage proteoglycan (section 2.2.6.2) by papain digestion and CPC precipitation from a solution 0.3 M in $MgCl_2$. The number average molecular weight ($\bar{M}n$) was calculated from the weight average molecular weight ($\bar{M}w$) determined by sedimentation equilibrium analysis using the $\bar{M}w/\bar{M}n$ ratio calculated from end group analysis with the NaB^3H_4 specific activity given by the manufacturers. $\bar{M}w$ from sedimentation equilibrium analyses was 24,400 and the $\bar{M}w/\bar{M}n$ ratio 1.127. Thus $\bar{M}n = 21,700$. This result is consistent with previously reported molecular weights for bovine nasal cartilage chondroitin sulphate (Luscombe and Phelps, 1967, Wasteson, 1969 and Robinson and Hopwood, 1973). The proteoglycan sample was digested with alkaline NaB^3H_4 as described previously and the specific activity determined in a manner similar to that described for the determination of molecular weight except that the unknown, in this case, is the specific activity.

Three separate determinations of specific activity gave 5.75×10^{12}, 5.64×10^{12}, and 5.56×10^{12} dpm/mole xylitol. The specific activity calculated from that reported by the manufacturers (Amersham) is 18.8×10^{12} dpm/mole $NaBH_4$ and would give 4.69×10^{12} dpm/mole xylitol and a molecular weight for the chondroitin sulphate of 17,500. A similar discrepancy in specific activities was also observed by Robinson and Hopwood (1973).

2.2.3.4 Calculation of Molecular Weight

Thus with a knowledge of the radioactivity in d.p.m., the weight of glycosaminoglycan and the specific activity of the NaB^3H_4, the molecular weight of the glycosaminoglycans can be calculated.

$$\text{mole} = \frac{\text{mass}}{\text{Mwt}}$$

where Mwt is the molecular weight of the sample and mass is the weight of the glycosaminoglycan in gm.

$$\text{mole} = \frac{\text{d.p.m.}}{5.64 \times 10^{12}}$$

where d.p.m. is the radioactivity of the end labelled sample and 5.64×10^{12} is the specific activity (in d.p.m./mole xylitol) of the NaB^3H_4. Thus -

$$\text{Mwt.} = \frac{\text{mass} \times 5.64 \times 10^{12}}{\text{d.p.m.}}$$

2.2.4 Gel Chromatography of Glycosaminoglycans

Aliquots of the glycosaminoglycans (about 0.5 mg/ml water or buffer) were applied to an analytical Sephadex G-200 column (90 x 1 cm) and eluted in an upward direction at 4 ml per hour with either pyridine acetic acid buffer (0.2 M in acetic acid) pH 5.0 or 0.2 M NaCl buffered with 0.02 M imidazole chloride pH 6.0. 2 ml fractions were collected and analysed for uronic acid and, in the case of alkaline tritiated borohydride treated sample, for radioactivity. Hyaluronidase digested or periodate oxidized and alkaline cleaved samples were chromatographed on the analytical G-200 column mentioned or on an analytical G-50 column (1 x 120 cm) in 0.2 M pyridine acetate buffer at pH 5.0 with upward elution at a rate of 4 ml per hour. The above columns were run at room temperature. The void volume of the columns was determined with blue dextran and the total volume determined with tritiated water.

2.2.5 Cellulose Acetate Electrophoresis of Glycosaminoglycans

Cellulose acetate electrophoresis was performed essentially as described by Habuchi et al (1973). Cellulose acetate strips (Sepraphore III) were soaked in pyridine; acetic acid; water (4:36:460) for several hours, lightly blotted, then placed in the electrophoresis tank. Sample (0.3 μg/μl) was applied to one side of the strip with a dual applicator (Gelman Instrument Co., Ann Arbor, Michigan, U.S.A.), and a standard mixture of glycosaminoglycan, containing 0.3 mg per ml of hyaluronic acid, 0.3 mg/ml of dermatan sulphate and 0.3 mg/ml of chondroitin 4- or 6-sulphate, was applied to the other side of the strip in the same operation. Electrophoresis was performed at a constant current of 0.5 mA per cm for one hour. The strips were blotted dry and stained with 0.5 percent alcian blue in 3% acetic acid for 20 min., followed by washing with 2% acetic acid for 1 min. then tap water for 10 min. Excess water was removed from the strips and they were placed in the clearing agent, 40 percent N-methyl pyrrolidone, for 5 min. They were then placed on clean glass slides and cleared at 80 to 90° C for 20 min. The slides were then scanned at 600 nm using a Beckman-Gilford spectrophotometer fitted with a Gilford linear transporter unit. Using this technique the mobility of one microgram of glycosaminoglycan could be compared accurately with that of a standard mixture.

2.2.6 Isolation and Purification of Proteoglycans

2.2.6.1 Cartilage Proteoglycans

Proteoglycan aggregate and subunit were prepared from milled cartilage as described by Hascall and Sajdera (1969) and Sajdera and Hascall (1969), (Fig. 6). Approximately 70 gm (wet weight) of milled cartilage was extracted with 1 litre of 4 M guanidinium chloride buffered to pH 5.8 with 0.05 M sodium acetate, at 4° C for 24 hrs. The extract was isolated by centrifugation at 23,000 g for 30 mins. and dialysed against 7 volumes of 0.05 M sodium acetate at pH 5.8 and 4° C, for 48 hrs. Solid cesium chloride was added to the dialysed extracts

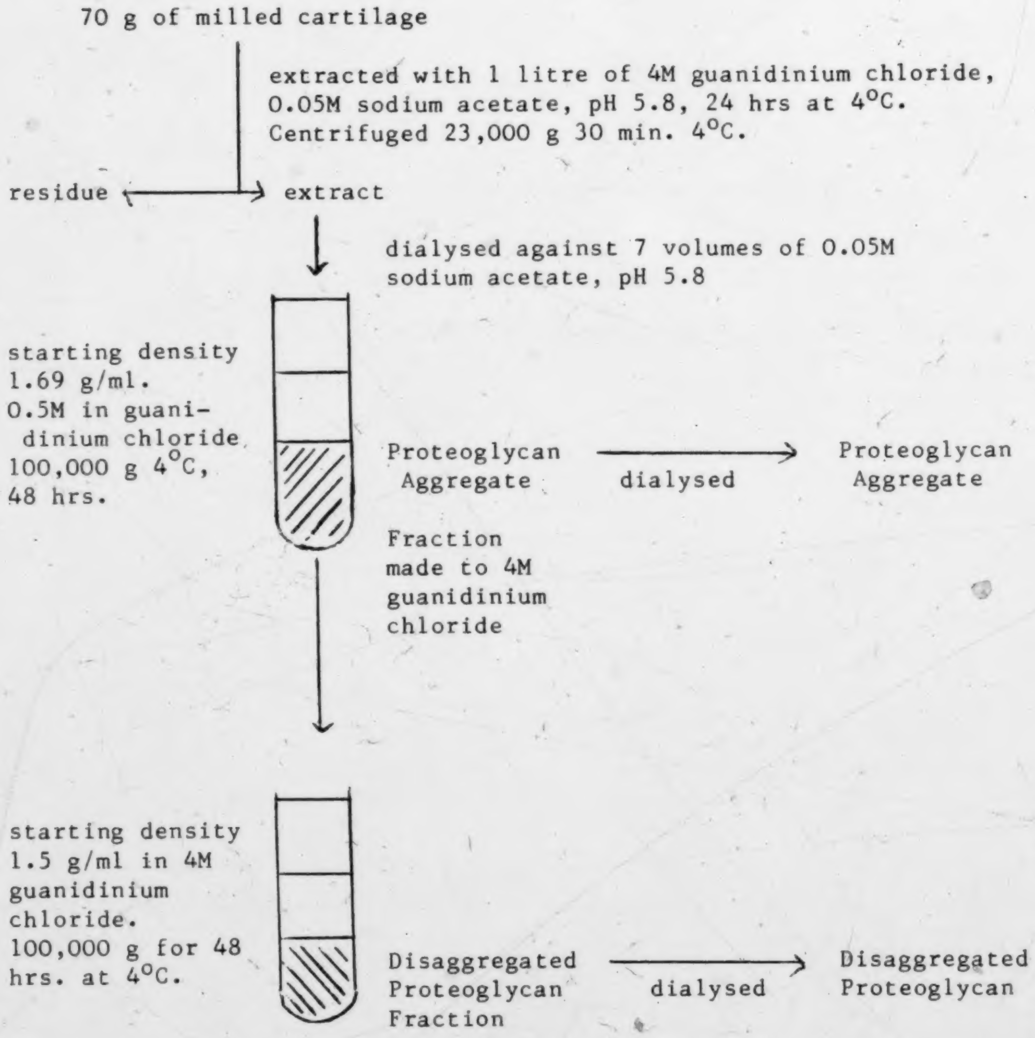

70 g of milled cartilage
extracted with 1 litre of 4M guanidinium chloride, 0.05M sodium acetate, pH 5.8, 24 hrs at 4°C. Centrifuged 23,000 g 30 min. 4°C.
residue
extract
dialysed against 7 volumes of 0.05M sodium acetate, pH 5.8
starting density 1.69 g/ml. 0.5M in guanidinium chloride 100,000 g 4°C, 48 hrs.
Proteoglycan Aggregate
dialysed
Proteoglycan Aggregate
Fraction made to 4M guanidinium chloride
starting density 1.5 g/ml in 4M guanidinium chloride. 100,000 g for 48 hrs. at 4°C.
Disaggregated Proteoglycan Fraction
dialysed
Disaggregated Proteoglycan

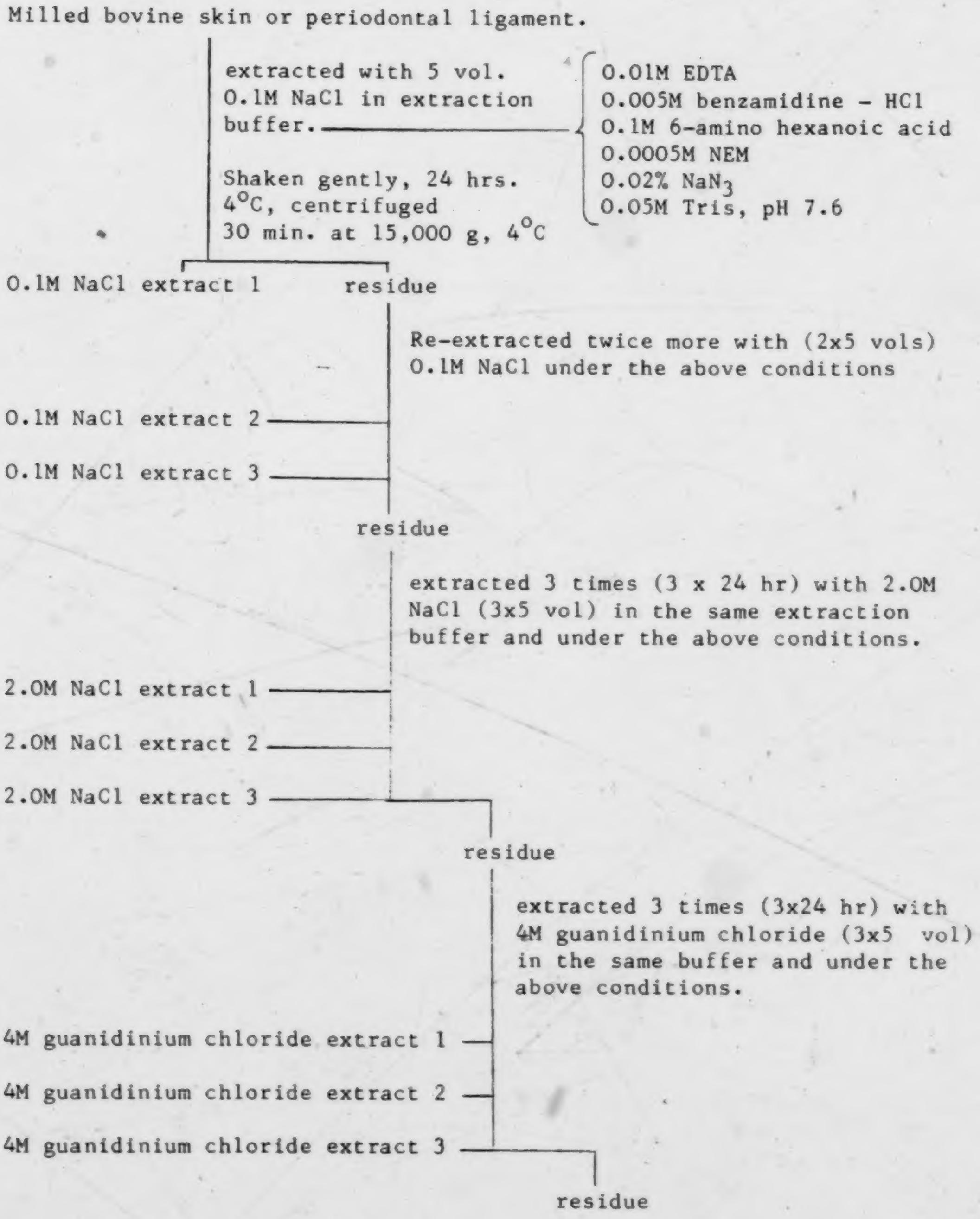
Fig. 6 The Preparation of Aggregated and Disaggregated Cartilage Proteoglycans

until the density reached 1.69 gm/l and a density gradient was formed by centrifugation at 100,000 g at 4° C for 48 hrs. The gradients were divided into three fractions, the bottom third of which contained proteoglycan aggregate. Half of this solution was dialysed at 4° C against several changes of distilled water (16 hrs.), then 1.0 M NaCl (48 hrs.) and finally 0.05 M sodium acetate, pH 5.8 (16 hrs.). The final solution was stored frozen and contained approximately 5 mg of proteoglycan aggregate per ml. The remaining half was made to 4 M guanidinium chloride by the addition of an equal volume of 7.5 M guanidinium chloride and to a density of 1.5 (gm/ml) with solid cesium chloride. This solution was centrifuged at 100,000 g for 48 hrs. at 4° C and the resulting gradients fractionated into thirds by aspiration from the top. The bottom third, containing approximately 90% of the total uronic acid and about 7 mg of disaggregated proteoglycan per ml, was dialysed extensively against distilled water, then 1 M NaCl and finally against 0.05 M sodium acetate, pH 5.8, at 4° C and stored frozen until required.

2.2.6.2 Non Cartilaginous Proteoglycans

2.2.6.2.1 Extraction

Proteoglycans from bovine skin and incisor periodontal ligament were isolated and purified essentially as described by Antonopoulos et al (1974) (Fig. 7). Frozen milled tissue (85 gm wet weight of periodontal ligament or a similar weight of skin) was extracted sequentially with 0.1 M NaCl, 2.0 M NaCl and 4 M guanidinium chloride. Five volumes of 0.1 M NaCl solution was added to the frozen milled tissue and shaken gently at 4° C for 24 hrs. The mixture was then centrifuged for 30 mins. at 4° C and 15,000 g. The supernatant was separated and the residue re-extracted with 5 volumes of 0.1 M NaCl solution. After extraction for 24 hrs. as described above the mixture was again centrifuged and the residue extracted once more with 0.1 M NaCl. This mixture was then centrifuged and the residue subjected to three sequential extractions with (5 volumes each) 2 M NaCl. The residue obtained from this extraction was then subjected to three sequential extractions with 4 M guanidinium chloride using the procedures described above. All extractions



Milled bovine skin or periodontal ligament.

extracted with 5 vol. 0.1M NaCl in extraction buffer.

- 0.01M EDTA
- 0.005M benzamidine - HCl
- 0.1M 6-amino hexanoic acid
- 0.0005M NEM
- 0.02% NaN_3
- 0.05M Tris, pH 7.6

Shaken gently, 24 hrs. 4°C, centrifuged 30 min. at 15,000 g, 4°C

0.1M NaCl extract 1

residue

Re-extracted twice more with (2x5 vols) 0.1M NaCl under the above conditions

0.1M NaCl extract 2

0.1M NaCl extract 3

residue

extracted 3 times (3 x 24 hr) with 2.0M NaCl (3x5 vol) in the same extraction buffer and under the above conditions.

2.0M NaCl extract 1

2.0M NaCl extract 2

2.0M NaCl extract 3

residue

extracted 3 times (3x24 hr) with 4M guanidinium chloride (3x5 vol) in the same buffer and under the above conditions.

4M guanidinium chloride extract 1

4M guanidinium chloride extract 2

4M guanidinium chloride extract 3

residue

Fig. 7 Extraction of Bovine Skin and Periodontal Ligament

Extraction of bovine skin did not include the 2M NaCl extractions.

were performed at 4° C and extraction solutions were buffered with 0.05 M Tris to pH 7.6 and contained 0.02 percent sodium azide as bacteriostatic agent and the following concentrations of protease inhibitors - 0.01 M ethylene diamine tetra acetic acid (EDTA), 0.005 M benzamidine hydrochloride, 0.1 M 6-aminohexanoic acid and 0.0005 M N-ethyl maleimide (NEM). All extracts were analysed for uronic acid, protein and hydroxyproline. Extraction of bovine skin differed slightly in that the 2 M NaCl extraction step was omitted and 1 mM PMSF was substituted for 0.1 M 6-aminohexanoic acid in the extraction buffers.

Due to the time and tedium of obtaining sufficient quantities of periodontal ligament for the extraction and purification of proteoglycans, initial experiments were conducted using bovine skin. In these experiments the skin was sequentially extracted with three treatments of 0.4 M guanidinium chloride followed by three treatments of 4.0 M guanidinium chloride. The conditions used were the same as described above (except that the pH of the 4 M guanidinium chloride solution was 5.8) and extracting solutions contained the same buffered protease inhibitor cocktail (containing 6-aminohexanoic acid).

2.2.6.2.2 Purification by DEAE - cellulose Chromatography

The three 0.1 M NaCl extracts were pooled as were the 2 M NaCl and 4 M guanidinium chloride extracts. These solutions were then concentrated to 50 to 100 ml on an Amicon PM 10 ultrafilter and the guanidinium chloride or sodium chloride was exchanged for a solution of 7 M urea in 0.05 M Tris buffer, pH 6.5 - 6.8, in the same apparatus. The solutions were then passed, at 4° C, through a 20 x 2.5 cm column of DEAE - cellulose which has been equilibrated with 7 M urea in 0.05 M Tris buffer, pH 6.5 - 6.8. The column was eluted with 2 - 3 bed volumes of 1) 7 M Urea, 2) 0.15 M NaCl in 7 M urea and 3) 2 M NaCl in 7 M urea at 45 ml/hour. All urea solutions were buffered to pH 6.5 - 6.8 with 0.05 M Tris and the fractionation was carried out at 4° C. The fractions were dialyzed extensively against distilled water, then against 1 M NaCl and finally against several changes of distilled water. The fractions were then lyophilized and stored at -40° C until required.

2.2.6.2.3 Density Gradient Purification

Density gradient centrifugation of these proteoglycans was performed essentially as described for cartilage proteoglycan (section 2.2.6.1) (Hascall and Sajdera, 1969). Initial experiments were performed on skin proteoglycans in order to determine the optimum starting density and conditions of centrifugation. Cesium chloride was dissolved in a guanidinium chloride-sodium acetate solution to give densities of 1.55 or 1.44 (gm/ml) and a final concentration of 4.0 or 0.4 M guanidinium chloride, 0.05 M sodium acetate, pH 5.8. Proteoglycan samples which had been extracted from bovine skin with 4 M guanidinium chloride and purified by DEAE - cellulose chromatography (2 M NaCl fractions) were dissolved in these solutions (approx. 1.5 mg/ml) and a density gradient was formed by centrifugation at 190,000 g at 4° C for 48 hrs. The tubes were divided into five equal fractions, using a Buchler tube piercer. Densities of the fractions were determined by using a 100 µl constriction pipette as a pycnometer and the fractions were analysed for uronic acid and protein.

Ligament proteoglycan, extracted with 4 M guanidinium chloride and purified by DEAE-cellulose chromatography, was dissolved in a solution of cesium chloride, 4 M guanidinium chloride and 0.05 M sodium acetate, pH 5.8 at an initial density of 1.41 gm/ml. A gradient was formed and analysed as described for the skin proteoglycan. Proteoglycan extracted from the periodontal ligament with 0.1 M NaCl solution and purified by DEAE-cellulose chromatography was subjected to the same gradient centrifugation, except that the starting density was 1.63 (gm/ml) and the guanidinium chloride concentration 0.5 M. The gradients were also divided into five fractions and analysed for uronic acid, protein and density.

2.2.7 Protease Digestion of Skin and Ligament Proteoglycans

25 µl solutions of bovine skin and periodontal ligament proteoglycans (4 mg/ml), purified by DEAE-cellulose chromatography, were mixed with 25 µl of buffer and 3 µl of stock protease solution and incubated at 37° C for 24 hrs. Control incubations were performed in the absence of enzyme. The buffer used for cathepsin D digestion was 0.2 M PAF,pH 3.6. That used for cathepsin B digestion was 0.1 M NaK phosphate, pH 6.0 and contained

1 mM EDTA and 1 mM cysteine. The buffer used for leucocytic elastase incubations was 0.05 M Tris HCl, 1 M KCl, pH 7.5. All incubations contained 0.02% NaN_3 final concentration. The products of protease digestion and control incubation were subjected to composite agarose polyacrylamide gel electrophoresis as described in section 2.2.10.

2.2.8 Incubation of Highly Purified Proteoglycans at 37° C

25 µl (aqueous solution) of 4 M guanidinium chloride extracted bovine skin and periodontal ligament proteoglycans (4 mg/ml) concentrated from the peaks (Fig. 24c and 25b) from Sepharose 6-B chromatography, previously purified by DEAE-cellulose chromatography and density gradient centrifugation were mixed with 25 µl of a) 0.05 M Tris-HCl, pH 7.2, 0.02% NaN_3, b) 0.05 M Tris-HCl 0.02% NaN_3, 0.2 M 6-aminohexanoic acid, pH 7.2 or c) 0.05 M Tris-HCl, 0.02% NaN_3, 1.8 mM phenylmethylsulphonyl fluoride (PMSF) and incubated at 37° C for 24 hrs. The products of incubation were subjected to composite agarose polyacrylamide gel electrophoresis as described in section 2.2.10.

2.2.9 Gel Chromatography of Proteoglycans

Proteoglycan samples (approximately 300 µg of uronic acid) were chromatographed on analytical Sepharose 2B, Sepharose 4B or Sepharose 6B columns (90 x 1 cm), eluted in an upwards direction with 0.5 M sodium acetate buffer pH 6.8 at 4.5 ml per hour and at 4° C. 2 ml fractions were collected and analysed for protein by absorbance at 280 nm and for uronic acid.

The void volume of the columns was determined with blue dextran or cartilage proteoglycan aggregate. The total volume of the columns was determined with tritiated water.

2.2.10 Composite Polyacrylamide-Agarose Gel Electrophoresis

Cylindrical gels containing 0.6 percent agarose and 2.0 percent (w/v) polyacrylamide were prepared by a method based on that of McDevitt and Muir (1971) and run under similar conditions. Stock buffer contained 0.2 M Tris-acetate containing 5 mM sodium sulphate adjusted at 4° C to pH 6.8 with acetic acid. The electrophoresis buffer was prepared by 20 fold dilution of this stock and made to 1 mM in EDTA. Stock acrylamide solution consisted of 29 percent acrylamide and 1 percent (w/v) bis-acrylamide and was made by adding 29.1 gm of acrylamide to 0.9 gm of N, N' methylene-bis-acrylamide. This solution was stored in the dark at 4° C. All chemicals used were Bio-Rad electrophoresis grade.

2.2.10.1 Gel Preparation

Agarose 0.18 gm, was added to 20 ml of boiling water in a measuring cylinder fitted with a cold finger condenser, and stirred vigorously at 100° C. After dissolution of the agarose (approximately 10 min.) 6 ml of stock buffer was added and the mixture allowed to cool to 43° C while being stirred. 2 ml of stock acrylamide solution was then added and the mixture maintained at 43° C and stirred. This solution was then transferred to an Erlenmeyer flask and 1.5 ml of freshly prepared ammonium persulphate (5 mg/ml) and 50 µl TEMED (N,N,N',N' - tetramethylenediamine, 0.17 percent) added. This solution was mixed and quickly transferred to glass electrophoresis tubes (13 cm x 0.6 cm i.d. and 0.1 cm wall thickness), previously sealed at one end with parafilm and placed vertically. The tubes were then transferred to the cold room to hasten gelation of the agarose and, after approximately 15 min., returned to room temperature and left to stand another 30 min. The gels were removed from the tubes and cut to exactly 10 cm to ensure a flat gel surface and consistent gel length. The gels were then carefully replaced in the glass tubes which were resealed at one end with dialysis tubing.

2.2.10.2 Electrophoresis Conditions

Proteoglycan samples were prepared by the combination of 5 µl of proteoglycan (4 µg/µl water) to 5 µl of 0.05 percent (w/v) bromphenol blue and 10 µl of 20 percent sucrose. This solution was layered on top of the gels under the electrophoresis buffer. A current of 1 mA per tube was applied until the bromphenol blue marker entered the gel (approximately 20 min.). It was then increased to 2 mA per tube for 40 min. and finally to 4 mA per tube for 60 min. Electrophoresis was performed in the cold room.

2.2.10.3 Localization of Bands

The gels were removed from their tubes and the position of the bromphenol blue dye marked with indian ink. The gels were then stained in 0.2 percent (w/v) toluidine blue in 0.1 M acetic acid for 30 min., for glycosaminoglycan chains either free or present as proteoglycans.

The gels were then destained in 3 percent acetic acid in a Bio-Rad diffusion destainer and scanned at 550 nm using a Gilford linear transporter on a Beckman DU Gilford spectrophotometer. Gels were also stained for protein by immersion for 16 hours in 0.045 percent Coomassie blue in 25 percent isopropanol, 10 percent acetic acid then in 0.0035 percent Coomassie blue in 10 percent isopropanol, 10 percent acetic acid for 6 hrs. The gels were destained in 10 percent acetic acid in a Bio-Rad diffusion destainer and scanned at 560 nm as described above.

2.2.11 Determination of Molecular Weights by Sedimentation Equilibrium

All experiments were performed at 20° C in a Beckman Model E ultracentrifuge equipped with both Schieren and Rayleigh interference optics. Velocity sedimentation was observed at 60,000 rpm. Molecular weights were determined either by the low speed equilibrium technique (Chervenka, 1969) and calculated as a function of concentration across the cell, using a program supplied by the Department of Biochemistry at this university, or by the meniscus depletion approach of Yphantis (1964). A 12 mm double sector cell was used with a charcoal filled epon centre piece and sapphire windows. The samples used for molecular weight determination were first dialysed for 48 hrs. against several changes of 0.1 M NaCl, 0.12 mM $NaHCO_3$, pH 6.8. A value of 0.52 was assumed for the partial specific volume of chondroitin sulphate (Robinson and Hopwood, 1973), 0.69 for the partial specific volume of the ligament proteoglycan (4 M guanidinium chloride extract) and 0.71 for the partial specific volume of the skin proteoglycan. The latter values were calculated from the buoyant density of the samples and were consistent with values obtained with very similar dermatan sulphate proteoglycans (partial specific volume 0.71, Preston, 1968) and also with the value obtained for keratan sulphate proteoglycans of similar polysaccharide content (45% protein, 55% polysaccharide, partial specific volume 0.67, Axelsson and Heinegård, 1978).

Weight average molecular weights of the cartilage chondroitin sulphate and the 4 M guanidinium chloride extracted skin proteoglycan (an aliquot of the fraction taken from Sepharose 6-B gel chromatography Fig. 24b) were determined by the low speed technique at concentrations of 1.42 and 1.31 mg/ml respectively and rotor speeds of 20,000 and 9,000 rpm respectively. The weight average molecular weight of the 4 M guanidinium chloride extract of the ligament proteoglycan (an aliquot of the fraction taken from Sepharose 6-B gel chromatography Fig. 25a) was determined by the meniscus depletion technique at a concentration of 0.5 mg/ml and a rotor speed of 15,000 rpm.

2.2.12 Chemical Estimations

2.2.12.1 Collagen

Collagen concentration was determined from hydroxyproline analyses (Stegeman and Stalder, 1967), assuming the hydroxyproline content of collagen to be 13.5 percent by weight.

2.2.12.2 Uronic Acid

Uronic acid analyses were performed by a modification (Bitter and Muir, 1962) of the Dische method (Dische, 1947) or by an automated modification of this technique (Heinegård, 1973). The uronic acid content of the various glycosaminoglycan fractions was determined after the fractions, precipitated with ethanol, were dried to constant weight at 70° C, over P_2O_5 in vacuo (section 2.2.3.2).

2.2.12.3 Protein

Protein was determined by the Lowry technique (Lowry et al, 1951) using bovine serum albumin as a standard.

2.2.12.4 Dermatan Sulphate and Iduronic Acid

Colorimetric dermatan sulphate determination was carried out by the Di Ferrante technique (Di Ferrante et al, 1971), using commercial dermatan sulphate (Miles) as a standard. Using this technique 40 µg dermatan sulphate gave an absorbance at 540 nm of approximately 0.45, whereas 500 µg of chondroitin 4-sulphate gave an absorbance of 0.035. I found the sensitivity of the assay to be critically dependant upon the quality of the Schiff reagent. The reagent was prepared by a technique similar to that described by Lillie (1965). 2 gm of basic fuchsin (Serva Feinbiochemica, Heidelberg) and 3.8 gm of sodium metabisulphite were dissolved in 100 ml of 0.25 M HCl. After 2 hours of gentle shaking the resultant solution, which was clear brown in colour, was decolourized by vigorous shaking with 1 gm of activated charcoal for 1 to 2 min. This mixture was filtered and the decolourization procedure repeated. The final solution which was water white was used within 12 hrs. of preparation. Glycosaminoglycan samples were also analysed for dermatan sulphate by hyaluronidase digestion as described in section 2.2.2.1. These two techniques agreed closely and for fifteen analyses of ligament glycosaminoglycans, where both techniques were used, varied by an average of 5%. The results obtained by the colorimetric technique tended to be slightly lower than that obtained by hyaluronidase digestion.

This colorimetric technique was also used to determine the proportion of iduronic acid to total uronic acid. This required a knowledge of the colour yield of iduronic acid as determined by the carbazole technique (Bitter and Muir, 1962) which was calculated as follows. 1 mg of standard chondroitin 4- or 6-sulphate was shown to contain 320 μg of uronic acid by the carbazole technique. However standard dermatan sulphate in which 80% of the uronic acid is iduronic acid (Inoue and Miyawaki, 1975), contained only 280 μg of uronic acid per mg as analyzed by this technique: a relative colour yield for dermatan sulphates identical to that obtained by Bitter and Muir (1962) for similar material. Thus the colour yield of iduronic acid can be calculated as follows:

$$320 \times 0.2 + 256x = 280$$

$$\text{i.e.} \quad 64 + 256x = 280$$

where 64 is the number of μg of glucuronic acid in 1 mg dermatan sulphate (i.e. 20% of 320) 256 is the number of μg of iduronic acid in 1 mg dermatan sulphate (i.e. 80% of 320), 280 is the number of μg of uronic acid obtained from carbazole analyses of 1 mg of dermatan sulphate, x is the colour yield of iduronic acid in the carbazole analyses, if the colour yield of glucuronic acid is taken as 1.0. This assumes that the colour yield of glucuronic acid in the carbazole reaction is not affected by the type of polymer in which it is contained.

Thus the colour yield of iduronic acid is calculated to be 0.844. The determination of iduronic acid by this technique is also complicated by the fact that only 80% of uronic acid in the standard dermatan sulphate used to make up standards for the colorimetric analyses was iduronic acid. Thus if UA is the number of µg of total uronic acid as determined by the carbazole technique, DS is the number of µg of dermatan sulphate as determined by the Di Ferrante technique, GUA is the number of µg of glucuronic acid and IUA is the number of µg of iduronic acid.

$$\mathrm{IUA} = \mathrm{DS} \ \mathrm{X} \ 0.256$$

Since the standard dermatan sulphate contains 256 µg iduronic acid per mg dry weight. Taking into account the contribution of iduronic acid to the carbazole uronic acid analysis:

$$\mathrm{UA} - 0.256 \ \mathrm{DS} \times 0.844 = \mathrm{GUA}$$

$$\frac{\mathrm{IUA}}{\mathrm{GUA} + \mathrm{IUA}} = \frac{\mathrm{DS} \times 0.256}{(\mathrm{UA} - 0.256 \times 0.844 \ \mathrm{DS}) + 0.256 \ \mathrm{DS}}$$

$$= \frac{\mathrm{DS} \times 0.256}{\mathrm{UA} + \mathrm{DS} \times 0.04}$$

Periodate will not oxidize 2-sulphated iduronic acid under these conditions, hence iduronic acid analysis by this technique excludes sulphated iduronic acid residues. Iduronic acid analysis by this technique also assumes the colour yield is unaffected by the composition of the glycosaminoglycan chain. Sulphated iduronic acid residues will further introduce errors in this estimation since the colour yield in the Bitter Muir uronic acid determination is also unknown.

2.2.12.5 Sulphate

Sulphate was determined by the rhodizonate technique of Terho and Hartiala (1971) with slight modification. The estimation depends on the formation of a coloured complex between barium and potassium rhodizonate. When inorganic sulphate is present, $BaSO_4$ forms and the colour intensity diminishes - the quantity of sulphate can be calculated from this reduction. $BaCl_2$ buffer solution was made up as follows: 10 ml of 2 M acetic acid, 2 ml of 0.005 M $BaCl_2$ and 8 ml of 0.02 M $NaHCO_3$ were made to 100 ml with absolute ethanol. $BaCl_2$ free buffer was made in the same way except that 2 ml of distilled water was substituted for the $BaCl_2$ solution. A solution of potassium rhodizonate was made by dissolving 6 mg (potassium rhodizonate, Aldrich Chemical Co.) in 20 ml water. 100 mg of L-ascorbic acid was then added and the solution shaken until all solids were dissolved. The volume was made up to 100 ml with ethanol. The solution, which was light brown in colour, was used after standing 30 min. and was not stable more than 24 hrs. Standard sulphate solutions, 1 to 8 µg sulphate were made from Na_2SO_4 in distilled water. All glassware was cleaned with nitric acid and rinsed repeatedly in distilled water. Two sets of standards and samples were used. $BaCl_2$ buffer was added to one set and $BaCl_2$ free buffer was added to the other set. It was noticed that in some instances a colour was formed in the absence of barium and the use of the barium free buffer was used to correct for nonspecific interference.

0.5 ml each of samples, standards and water were pipetted into test tubes and 2 ml ethanol was added. 1.0 ml of barium chloride buffer was added to another set of standards and samples. 1.5 ml of potassium rhodizonate solution was added and the tubes shaken well. It was important to add the rhodizonate solution immediately after the $BaCl_2$ buffer. Failure to do this resulted in very erratic absorbance readings. The absorbance at 520 nm was read after allowing the tubes to stand for 10 min. at room temperature.

A standard curve was constructed by subtracting the absorbance of a given sulphate standard from that of the water sample and plotting this difference in absorbance against the µg sulphate present. The standard curve is linear and allows the determination of between 1 and 6 µg of sulphate. Calculation of the sulphate contents of the samples was performed by subtracting the absorbance value found in the absence of Ba from that of the sample with Ba. This value was subtracted from the absorbance of distilled water and the amount of sulphate corresponding to this value was found from the standard curve. In most cases the Ba free control had a negligible absorbance.

2.2.12.6 Radioactivity

Radioactivity was assayed using a Searle Mark III liquid scintillation system. Aqueous samples were mixed with 10 ml of Unisolve 1 (Koch Light Laboratories Ltd., Bucks, England).

2.2.12.7 Hexosamines

Glucosamine and galactosamine were determined by ion exchange chromatography after hydrolysis in 4 M HCl at 100° C for 18 hours. The acid was removed in a vacuum desiccator over P_2O_5 and NaOH.

A column of Chromobeads A resin (Technicon) was employed, jacketed at 60° C and the separated amino sugars were determined with ninhydrin using a Technicon apparatus (Pearson et al, 1978a). A 45 x 0.6 cm column and a citrate buffer gradient pH5 to pH4.5 was employed in some analyses, as described by Pearson et al (1978a) for total hydroxylysine determinations.

In later work an 11 x 0.6 cm column was preferred, eluting with a single buffer (sodium citrate, 0.1 M, pH5.0).

Hydrolysates were dissolved in 1 - 3 mls of 0.1 M citrate buffer and 1.0 ml containing 20 - 50 µg of hexosamine (45 cm column) or 10 - 25 µg hexosamine (11 cm column) was analysed. L-leucine (Sigma) was run just before the sample as a standard. Colour yields of both hexosamines were as given for glucosamine by Pearson et al,(1978a).

2.2.12.8 Amino Acid Analyses

Samples for analyses were dried on an Buchler evapomix and dissolved in constant boiling 6 M HCl. A crystal of phenol was added to reduce oxidation, the solutions were bubbled with nitrogen and then sealed and incubated at 110° C for 21 hrs. The hydrolysed samples were dried quickly on a Buchler evapomix and dissolved in distilled water. The amino acid content was determined on a Beckman 121 MB automatic amino acid analyser using the single column collagen hydrolysate procedure with a Beckman AA-10 resin. Methionine was determined as the sulphoxide. The phenylalanine peak was largely obscured by the presence of a large hexosamine peak in this system. The phenylalanine results quoted are thus only estimates possibly containing large errors.

2.2.13 Histology

Bovine incisors of various stages of development were fixed and decalcified in 60 percent formalin containing 10 percent EDTA and 4% cetyltrimethylammonium bromide (CTAB). After decalcification the tissues were embedded in paraffin and sectioned by routine procedures. The sections were stained with hematoxylin-eosin, Masson trichrome or Van Giesens stain. Some sections were stained for glycosaminoglycans with alcian blue, using the differential staining technique of Scott and Dorling (1965).

CHAPTER 3

THE GLYCOSAMINOGLYCANS OF THE PERIODONTAL LIGAMENT OF THE MATURE OCCLUDED BOVINE INCISOR

3.1 GROSS AND HISTOLOGICAL APPEARANCE OF THE INCISOR PERIODONTAL LIGAMENT

The periodontal ligament from fully occluded bovine incisors was tough and fibrous and firmly attached to both the root surface and the alveolar bone. Attachment to the root surface seemed stronger since it usually remained attached when the tooth was removed from the fractured mandible. Histological examination of the ligament revealed dense bundles of wavy collagen fibres, the majority of which are oriented obliquely across the periodontal space. The continuity of these collagen fibres between the cement and the periodontal ligament was particularly noticeable (Plate 3). Between these were collections of loose, less fibrous connective tissue with which blood vessels are frequently associated. The proteoglycans of the matrix appear largely sulphated as revealed by differential alcian blue staining (Scott and Dorling, 1965) and appear to be associated with the collagen bundles.

3.2 TECHNIQUES OF GLYCOSAMINOGLYCAN ANALYSIS IN GENERAL

The wide variety of techniques employed to analyse glycosaminoglycans can be classified into two broad groups - the degradative techniques such as those employing enzymes (e.g. the chondroitinases and hyaluronidase) or degradative chemical techniques (e.g. controlled periodate oxidation) specific to certain disaccharide or monosaccharide structures; and the nondegradative techniques, those which separate glycosaminoglycans by physical techniques, which are based largely on differences between the various polysaccharides with regard to charge density and solubility in ethanol. These include such methods as electrophoresis, ion exchange chromatography, fractionation of quaternary ammonium complexes as well as ethanol precipitation.

Both types of technique have their advantages and disadvantages. For example, although resistance to hyaluronidase digestion has been regarded as one of the characteristic properties of dermatan sulphate, the finding that this polysaccharide is a hybrid containing a certain amount of glucuronic acid has led to a revision of this notion. The presence of the glucuronic acid makes the molecule susceptible to attack by hyaluronidase at the hexosaminic bonds involving these uronic acid units, and a partial degradation may therefore occur. Nevertheless, the use of hyaluronidase and more recently the chondroitinases enables the convenient differentiation between very small amounts of dermatan sulphate type and chondroitin sulphate type glycosaminoglycans. The physical techniques, although producing no degradation of the polysaccharides, seldom give complete separation of a complex glycosaminoglycan mixture. Thus a combination of techniques is usually employed. Fractionation of the quaternary ammonium complexes (CPC precipitation) in combination with hyaluronidase digestion, controlled periodate oxidation linked with the colorimetric determination of released aldehyde, or alcohol precipitation from acetic acid - calcium acetate solutions, was used to analyse or fractionate the glycosaminoglycans present in the bovine periodontal ligament. The efficiency of these techniques was first tested on a mixture of standard glycosaminoglycans.

3.3 ANALYSIS OF THE PERIODONTAL LIGAMENT GLYCOSAMINOGLYCANS FRACTIONATED BY CPC PRECIPITATION

Polyanions form water-insoluble salts with certain detergent cations such as cetylpyridinium (CP). The complexes are dissociated and dissolved by inorganic salts at certain concentrations (critical electrolyte concentrations) which depend largely on the charge density of the polymer (Scott, 1960).

1 mg each of hyaluronic acid, chondroitin 4-sulphate, chondroitin 6-sulphate and dermatan sulphate was fractionated by CPC precipitation as described in section 2.2.1.1 . The recovery of glycosaminoglycan in each fraction is shown in Table 6.

As expected from previous work (Scott, 1960) hyaluronic acid was recovered from the 0.03 M NaCl fraction while the sulphated glycosaminoglycans were recovered in the 0.3 M $MgCl_2$ fraction. Recoveries better than 85% were routinely obtained.

	percentage of the total uronic acid in each fraction			total % recovery
	0.03 M NaCl	0.15 M $MgCl_2$	0.3 M $MgCl_2$	
Hyaluronic acid	90.2	0.1	2.7	93
Chondroitin 4-sulphate	1.4	1.7	89.3	92
Chondroitin 6-sulphate	0.9	1.4	84.7	87
Dermatan sulphate	7.0	0.4	84.5	92

Table 6. Recovery of Standard Glycosaminoglycans after CPC Precipitation

1 mg of each glycosaminoglycan was fractionated by CPC precipitation. 0.03 M NaCl refers to that fraction precipitated by CPC from 0.03 M NaCl but remaining in the supernatant after CPC precipitation in 0.15 M $MgCl_2$. 0.15 M $MgCl_2$ refers to that fraction precipitated from 0.15 M $MgCl_2$ by CPC but remaining in solution in 0.3 M $MgCl_2$. 0.3 M $MgCl_2$ refers to that fraction precipitated from a solution of 0.3 M $MgCl_2$ by CPC.

In order to separate chondroitin sulphate from dermatan sulphate, the 0.3 M $MgCl_2$ fraction from the CPC precipitation of a mixture of glycosaminoglycans was digested with hyaluronidase. The recovery of uronic acid in the various fractions is shown in Table 7. If correction is made for the digestion of standard dermatan sulphate shown to be 20 percent, good recovery and fractionation of the glycosaminoglycans is obtained. Furthermore these results show that hyaluronidase digestion was unaffected by prior CPC precipitation.

Glycosaminoglycans were isolated from mature bovine incisor ligament by papain digestion and fractionated with CPC as described in section 2.2.1.2 . The dermatan sulphate content of the 0.3 M $MgCl_2$ fraction was determined by hyaluronidase digestion and/or by the colorimetric analyses of Di Ferrante (Di Ferrante _et al_, 1971). The results in Table 8 show that the majority of the ligament glycosaminoglycans are of the chondroitin sulphate (31% of total glycosaminoglycan) and dermatan sulphate (43% of the total glycosaminoglycans) types. There is also a significant amount of hyaluronic acid (24%) and a small proportion (2%) of an intermediate fraction (0.15 M $MgCl_2$ fraction).

GAG	µg uronic acid					% total recovery
	0.03 M NaCl fraction	0.3 M $MgCl_2$ fraction				
		HA'se CPC supernatant	CS fraction	HA'se CPC precipitate	DS fraction	
1	204.8	79.4	68.5	43.6	54.5	100
2	82.0	90.6	59.8	123.2	154.0	104

Table 7. Recovery of Glycosaminoglycans after Hyaluronidase Digestion

Two mixtures of standard glycosaminoglycans (GAG) containing 1.204 µg hyaluronic acid, 67.2 µg chondroitin sulphate and 54.4 µg dermatan sulphate, 2. 81.8 µg hyaluronic acid, 67.2 µg chondroitin sulphate and 136 µg dermatan sulphate (expressed as µg in uronic acid) were fractionated by CPC precipitation and digested with hyaluronidase. HA'se CPC supernatant refers to the digested glycosaminoglycan not precipitable with CPC. HA'se CPC precipitate refers to the material precipitating with CPC after hyaluronidase digestion. The CS (chondroitin sulphate) and DS (dermatan sulphate) fractions were obtained by correcting for the hyaluronidase digestion (20%) of standard dermatan sulphate.

The homogeneity of each fraction was assessed by cellulose acetate electrophoresis. As shown in Fig. 8, the hyaluronic acid fraction ran as a sharp band with a very similar mobility to that of a standard hyaluronic acid. The 0.15 M $MgCl_2$ fraction ran as a broader band with a mobility between standard hyaluronate and dermatan sulphate. The sulphated glycosaminoglycan fraction (0.3 M $MgCl_2$ fraction) migrated as a broad band, the mobility of which extends from slightly less than that of standard dermatan sulphate to slightly less than that of standard chondroitin sulphate. After digestion with hyaluronidase the dermatan sulphate fraction migrated as a sharp band, with a mobility slightly less than that of the standard dermatan sulphate.

In order to investigate the nature of the 0.15 M $MgCl_2$ fraction it was subjected to sequential hyaluronidase and chondroitinase ABC digestion. This produced no noticeable change in behaviour on cellulose acetate electrophoresis, though a partial digestion of the glycosaminoglycan mixture could have occurred, since the electrophoresis results were not quantitated. A standard mixture of glycosaminoglycans

Fraction	µg uronic acid / mg hyp	percent dry weight
Hyaluronic Acid	7.05 ± 0.61	0.127 ± 0.013
Chondroitin Sulphate	9.0 ± 2.3	0.205 ± 0.05
Dermatan Sulphate	12.6 ± 2.2	0.332 ± 0.05
0.15 M $MgCl_2$ precipitate	0.61 ± 0.09	

Table 8. Glycosaminoglycan Composition of Bovine Periodontal Ligament from Mature Occluded Incisors

Bovine periodontal ligament from mature occluded incisors was digested with papain and the glycosaminoglycans released fractionated with CPC. The sulphated glycosaminoglycan fraction (that precipitated from 0.3 M $MgCl_2$ by CPC) was analysed for dermatan sulphate and chondroitin sulphate by hyaluronidase digestion or for dermatan sulphate by the Di Ferrante technique. In the latter case chondroitin sulphate was determined by difference. The values given are the averages and standard deviations from 7 separate analyses of individual or pooled ligaments. The uronic acid composition of standard hyaluronic acid, chondroitin sulphate, and dermatan sulphate was calculated from analyses of dry samples, to be 41%, 32% and 28% respectively. These figures were used in the calculation of the weight of the ligament glycosaminoglycan fractions.

(containing hyaluronic acid, chondroitin 4-sulphate and dermatan sulphate) after similar digestion could not be detected on cellulose acetate electrophoresis and was assumed to be completely digested to disaccharides. The enzyme digested 0.15 M $MgCl_2$ fraction was then subjected to digestion with nitrous acid as described in section 2.2.2.4. The material, thus treated, could not be detected by alcian blue staining after cellulose acetate electrophoresis, and was assumed to be completely degraded. Nitrous acid digestion produced only slight band broadening with a mixture of standard glycosaminoglycans. Nitrous acid reacts with the free amino groups and the N-sulphated hexosamine units occurring in heparin and heparan sulphate. Susceptible glucosamine residues are converted to 2, 5-anhydro-D-mannose during the reaction and concurrent cleavage of adjacent glycosidic bonds occurs (Lagunoff and Warren, 1962). Thus the behaviour of the 0.15 M $MgCl_2$ fraction was considered typical of heparin and heparan sulphate. However, since the ratio of glucosamine to galactosamine in this fraction was found to be only 1:2 (Table 10.), this fraction was

Plate 1. Cellulose Acetate Electrophoresis of the Galactosaminoglycan Fractions of the Mature Incisor Periodontal Ligament

The 18, 25, 30, 40 and 50% alcohol fractions of the periodontal ligament galactosaminoglycans were subjected to cellulose acetate electrophoresis as described in Fig. 8. The cleared strips are shown. CS, DS and HA, standard chondroitin sulphate, dermatan sulphate, and hyaluronic acid. The broad bands and inability to resolve the 40 and 50% alcohol fractions are believed due to their copolymeric nature (Fransson and Rodén, 1967a), though effects due to possible variations in the degree of sulphation cannot be eliminated.

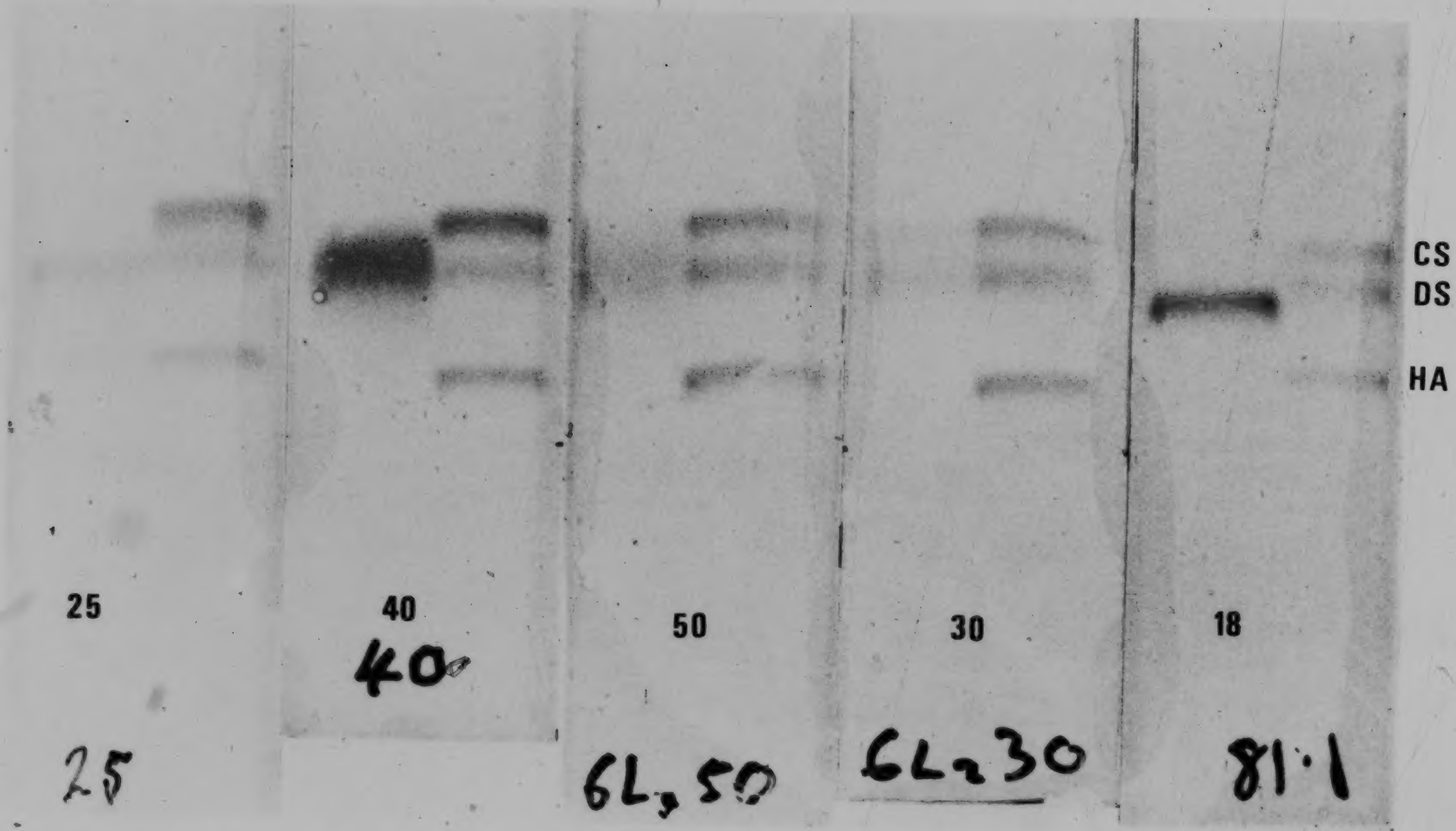
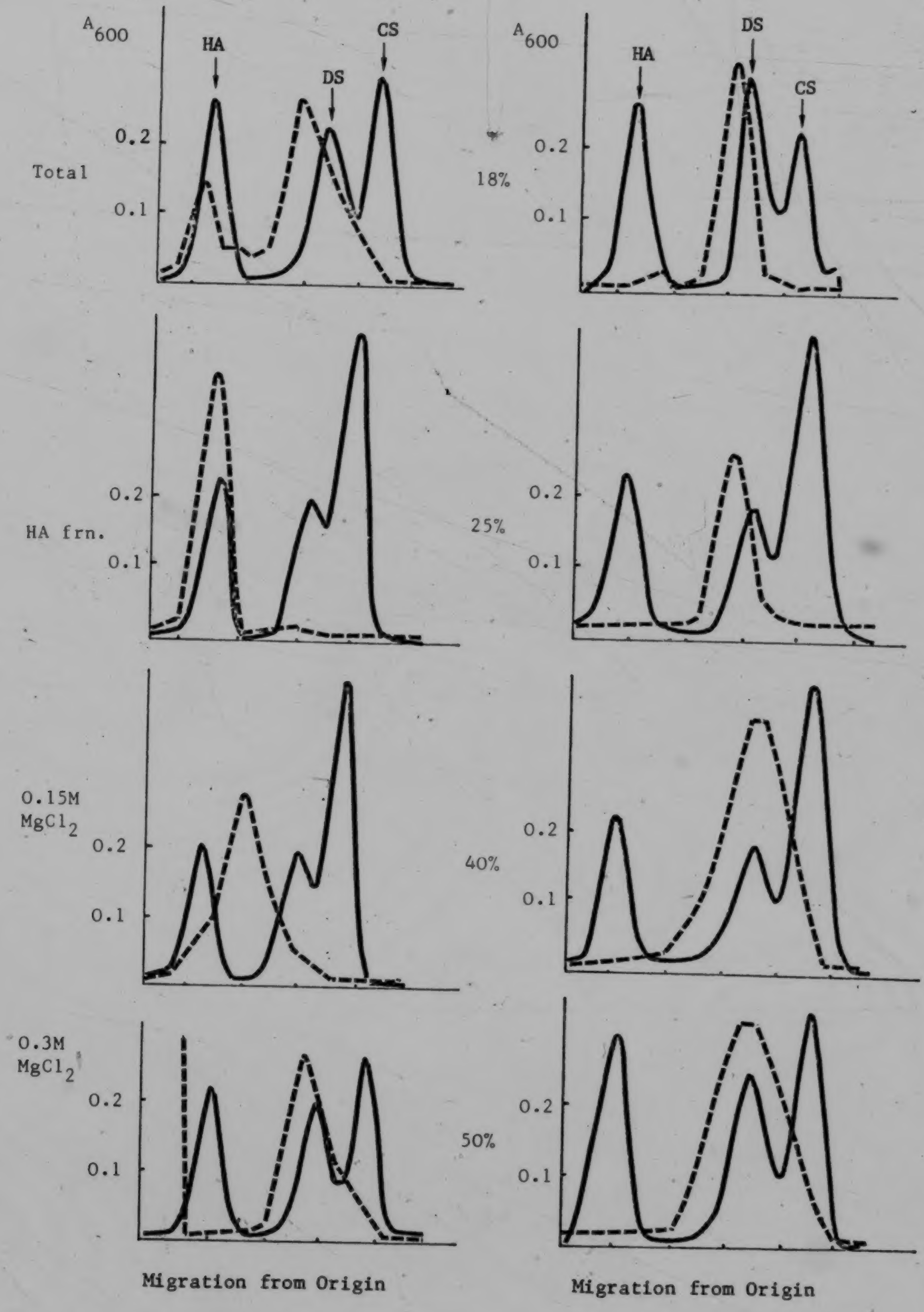

CS
DS
HA
25
40
50
30
18
25
40
6L,50
6L,30
81·1

Fig. 8. Densitometric Scans of Cellulose Acetate Electrophoresis of Ligament Glycosaminoglycans

Ligament glycosaminoglycan samples (1.0 µg/µl) were applied to one side of cellulose acetate strips. Simultaneously a mixture of standard glycosaminoglycans (1.0 µg/µl hyaluronic acid, 1.0 µg/µl dermatan sulphate, 1.0 µg/µl chondroitin 4-sulphate and/or 1.0 µg/µl chondroitin 6-sulphate) was added to the other side of the strip with a dual applicator. Electrophoresis was then performed in pyridine: acetic acid: H_2O (1:9:115), at a constant current of 0.5 mA/cm for 1 hr. The strips were stained with alcian blue and cleared. Each side of the clear strip was scanned separately at 600 nm and the standard and sample profile superimposed. Total represents the unfractionated periodontal ligament glycosaminoglycans. HA frn, the hyaluronate fraction precipitated with CPC from 0.03 M NaCl. 0.15 M $MgCl_2$ and 0.3 M $MgCl_2$ are the fractions precipitated from 0.15 M and 0.3 M $MgCl_2$ respectively. 18%, 25%, 40% and 50% are the fractions precipitated from 18, 25, 40 and 50% ethanol. HA, DS and CS indicate the position of standard hyaluronic acid, dermatan sulphate and chondroitin sulphate respectively. The solid lines are the profiles of the standard glycosaminoglycan mixtures and the dotted lines the profiles of ligament glycosaminoglycan fractions.


A_{600}
HA
DS
CS
0.2
0.1
Total
HA frn.
0.15M $MgCl_2$
0.3M $MgCl_2$
18%
25%
40%
50%
Migration from Origin
Migration from Origin

assumed to contain a mixture of glycosaminoglycans - probably an undersulphated chondroitin sulphate as well as heparan sulphate and appears very similar to a fraction (F.3) isolated from the periodontal ligament by Pearson et al (1975).

Fractionation of CPC complexes of standard glycosaminoglycans on cellulose columns by the technique of Tanaka and Gore (1966) was also investigated, however, this technique was abandoned after many trials due to very poor recoveries and inconsistent separation of standard glycosaminoglycan mixtures.

3.4 ANALYSIS OF THE PERIODONTAL LIGAMENT GALACTOSAMINOGLYCANS FRACTIONATED BY ALCOHOL PRECIPITATION

3.4.1 Chemical Analysis

Fractional precipitation with ethanol is one of the classical methods for the separation of polysaccharide mixtures and gives efficient separation of very similar glycosaminoglycans (in particular the chondroitin sulphates and dermatan sulphate) which are extremely difficult to resolve by other nondegradative procedures.

1.0 mg each of chondroitin 4-sulphate, chondroitin 6-sulphate and dermatan sulphate was fractionated by alcohol precipitation and the recovery of uronic acid in the various fractions is shown in Table 9. Excellent recovery and a distinct separation of the standard glycosaminoglycans was obtained using this technique. The presence of some uronic acid in the 30 and 40 percent ethanol fractions of the dermatan sulphate may be explained by the presence of small amounts of glycosaminoglycans which contain a larger proportion of glucuronic acid (Fransson and Rodén, 1967a).

The 0.3 M $MgCl_2$ fraction obtained by CPC precipitation of the ligament glycosaminoglycans was further fractionated using this technique and, as can be seen from Table 10, fractionation of glycosaminoglycans with different iduronate contents was achieved. Although there is a progressive decrease in iduronic acid content with increasing ethanol concentration, the fractions can be grouped into approximately equal proportions of two species of glycosaminoglycans, one rich in iduronic acid - the 18 and 25% ethanol fractions containing

ethanol fraction	% uronic acid					% total recovery
	18	25	30	40	50	
glycosaminoglycan						
chondroitin 4-sulphate	1.4	1.6	23.8	62.2	11.0	92
chondroitin 6-sulphate	1.8	1.4	1.0	83.5	12.2	100
dermatan sulphate	54.9	28.2	9.5	6.1	1.2	100

Table 9. Alcohol Fractionation of Standard Glycosaminoglycans

1 mg each, of chondroitin 4-sulphate, chondroitin 6-sulphate and dermatan sulphate, was fractionated by alcohol precipitation in calcium acetate-acetic acid. Fractions were obtained at 18,25, 30, 40 and 50 percent ethanol concentrations.

90.2 and 73.5% of the total uronic acid as iduronic acid respectively and one rich in glucuronic acid - the 40 and 50% ethanol fractions containing 84.4 and 94.7% of the total uronic acid as glucuronic acid respectively. Electrophoretic profiles on cellulose acetate strips shown in Plate 1 and densitometric scans of these strips in Fig. 8 support this grouping. The 18 and 25% ethanol fractions migrated as single sharp bands with mobilities slightly less than that of the standard dermatan sulphate. The reason for the consistently lower mobility of these fractions compared with the standard dermatan sulphate is unknown but may involve differences in glucuronate content or distribution. The 40 and 50% ethanol fractions migrated as broad bands with an average mobility between that of the chondroitin sulphate and dermatan sulphate standards. Glucosamine could not be detected in these fractions (Table 10.) suggesting they were free from contamination with hyaluronic acid and the heparin type glycosaminoglycans (as was also indicated by the cellulose acetate electrophoresis).

The fractions show little difference in the degree of sulphation, however, differences in the position of sulphation were demonstrated in disaccharides obtained by chondroitinase digestion. The fractions were digested and the disaccharides produced separated by paper chromatography. Predominantly one disaccharide was obtained from the 18 and 25% fractions corresponding to the Δdi-4S disaccharide produced by digestion of chondroitin 4-sulphate (Yamagata et al, 1968). A small amount (19% of the total disaccharides) of material corresponding to

fraction	% dry weight	µg UA / mg hyp	IdUA %	$GlNH_3$ µg/mg hyp	$GalNH_3$ µg/mg hyp	SO_4: $hexNH_2$ molar ratio	4-sulphate %	6-sulphate %
HA	0.127 ± 0.013	7.1 ± 0.6	0	5.61	0	0		
0.15 M $MgCl_2$		0.61 ± 0.09		0.128	.254			
18	0.164 ± 0.020	6.8 ± 0.7	90 ± 5	0	6.46	1.1	98	2
25	0.055 ± 0.004	2.2 ± 0.2	74 ± 3				100	0
40	0.108 ± 0.014	5.0 ± 0.7	16 ± 3	0	3.35	1.1	81	19
50	0.110 ± 0.014	5.2 ± 0.8	5 ± 3	0	3.39	0.95	52	48

Table 10. Analyses of Glycosaminoglycan Fractions from the Periodontal Ligament of the Mature Occluded Bovine Incisor

The glycosaminoglycan fractions from ligaments of mature occluded bovine incisors were prepared by papain digestion followed by CPC precipitation and ethanol precipitation from calcium acetate-acetic acid. The weight of the fractions was determined from uronic acid analyses using a conversion factor (i.e. mg uronic acid/mg dry weight of glycosaminoglycan) determined previously. The analyses given for percentage dry weight, µg UA (uronic acid) per mg hydroxyproline and iduronic acid (IdUA) are the average of eight separate analyses of ligament or pooled ligaments (except fractions HA and 0.15 M $MgCl_2$ which are averages of 7 and 5 analyses respectively) and include standard deviations. Iduronic acid was determined by the Di Ferrante technique and is expressed as a percentage of total uronic acid. Glucosamine ($GlNH_3$) and galactosamine ($GalNH_3$) were separated after hydrolysis in 4 M HCl at 100° C for 18 hrs. as described in section 2.2.12.7. Total sulphate was determined by the rhodizonate technique (Terho and Hartiala, 1971). N-acetyl galactosamine 4- and 6-sulphates were separated as disaccharides and quantitated as described by Saito et al (1968). Each is expressed as a percentage of total 4- and 6-sulphate, HA refers to the hyaluronic acid fraction obtained by CPC precipitation from 0.03 M NaCl. The 0.15 M $MgCl_2$ fraction is that material precipitated from 0.15 $MgCl_2$ with CPC. Fractions 18, 25, 40 and 50 were obtained by precipitation of the 0.3 M $MgCl_2$ fraction at ethanol concentrations of 18, 25, 40 and 50 percent. The 0.3 M $MgCl_2$ fraction was the glycosaminoglycan obtained by CPC precipitation from 0.3 M $MgCl_2$. 0 refers to analyses below the limits of quantitation.

the Δdi-6S was apparent in digests of the 40% alcohol fractions, whereas digests of the 50% fraction showed approximately equal proportions of Δdi-4S and Δdi-6S disaccharides (Table. 10.). Whereas the standard glycosaminoglycan mixtures (dermatan sulphate, chondroitin 4-sulphate and chondroitin 6-sulphate, Miles Research Products) were completely digested (greater than 95%) to disaccharides, digestion of the alcohol fractions of the ligament glycosaminoglycans appeared to be less complete. Approximately 15 to 30% of the polymers remained near the origin after paper chromatography of the chondroitinase ABC digests. The 18% ethanol fraction consistently showed the least digestion. These results can be explained by the presence of regions of polymer containing sulphated iduronic acid residues. Iduronic acid sulphate containing oligosaccharides are known to be poor substrates for chondroitinase ABC (Fransson et al, 1974 and Cöster et al, 1975).

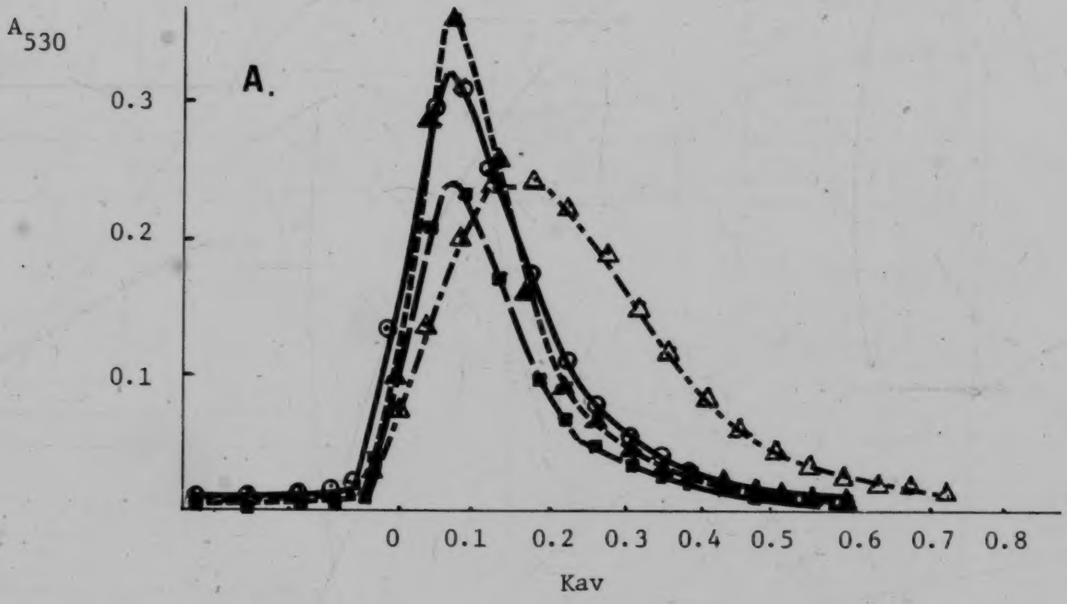
3.4.2 Gel Chromatography

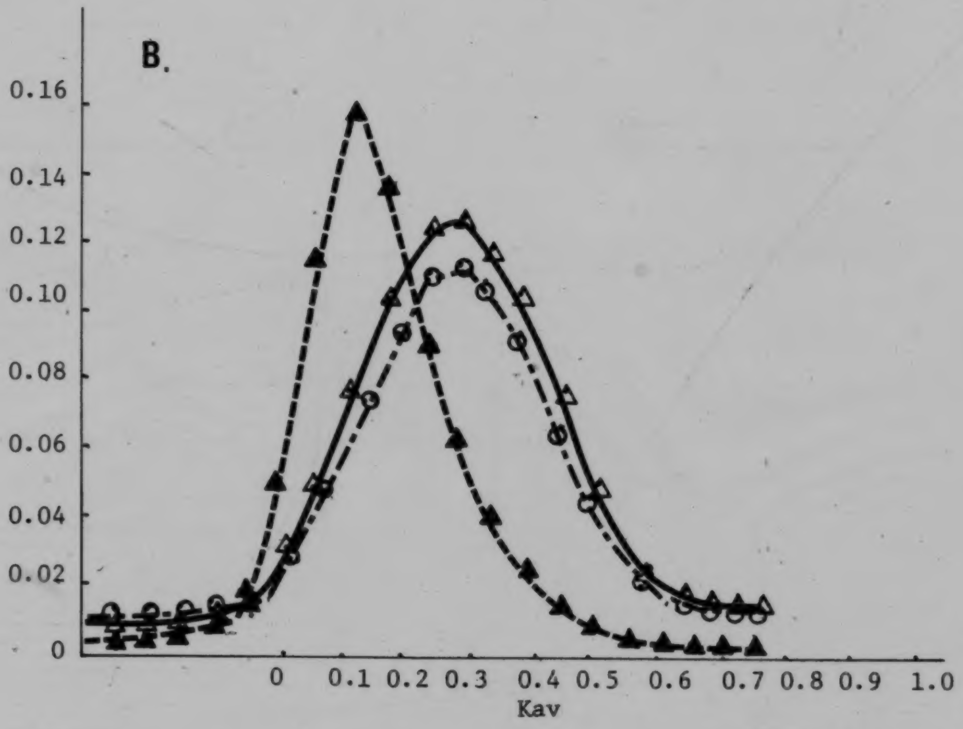
Fig. 9 shows gel chromatography profiles on Sephadex G-200 of glycosaminoglycan fractions from the periodontal ligament. The 18, 25 and 40% alcohol fractions have almost identical elution behaviour and appear to be much less polydisperse than the 50% ethanol fraction which has an elution behaviour almost identical to that of cartilage chondroitin sulphate. Since it has been shown that the glycosaminoglycan chain configuration and thus elution behaviour is greatly influenced by the type and concentration of cation (Mathews, 1953, Mathews, 1956 and Urist et al, 1968), it was suggested that the pyridinium cation may have influenced elution behaviour of these glycosaminoglycans. However elution with 0.2 M buffered sodium chloride made very little difference to the elution profiles of the ligament glycosaminoglycan fractions. The 18, 25 and 40% ethanol fractions again had essentially identical elution behaviour (the 25 and 40% fractions are not shown in Fig. 9b for the sake of simplicity), while the 50% fraction and cartilage chondroitin sulphate appeared smaller and much more polydisperse. Elution behaviour was also unchanged after incubation in 1.0 M NaOH for 16 hrs. at 4° C suggesting that the polymers are not peptidoglycans with multiple dermatan sulphate or chondroitin sulphate chains attached

Fig. 9. Gel Chromatography of the Alcohol Fractions of Ligament Glycosaminoglycans on Sephadex G-200

a) Samples of the alcohol fractions of the ligament glycosaminoglycans, approximately 1 mg dissolved in 0.2 M pyridine acetic acid buffer, pH 5.0, were applied to a Sephadex G-200 column (90 x 1.0 cm) and eluted with the same buffer. 2 ml fractions were collected and analysed for uronic acid. (○) 40% alcohol fraction, (▲) 18% alcohol fraction, (■) 25% alcohol fraction, (△) 50% alcohol fraction.

b) Samples (0.3mg) of the alcohol fractions of the ligament glycosaminoglycans and a chondroitin sulphate isolated from cartilage proteoglycan by alkaline cleavage and precipitation in 40% ethanol (see section 3.4.3) were chromatographed on Sephadex G-200 as described in a), except that the column was equilibrated and eluted with 0.2 M NaCl, buffered with 0.02 M imidazole chloride, pH 6.0. (△) cartilage chondroitin sulphate, (▲) 18% ethanol fraction, (○) 50% ethanol fraction.


A_{530}
A.
0.3
0.2
0.1
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
Kav


B.
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
Kav

to single polypeptide cores.

3.4.3 Molecular Weight Determinations

In order to calculate their molecular weight and examine their molecular weight distribution, the 18%, 40% and 50% alcohol fractions of glycosaminoglycans isolated from the periodontal ligament were subjected to alkaline cleavage and reduction with tritiated sodium borohydride. The molecular weights of the glycosaminoglycan fractions, calculated from these experiments, were consistently very high for glycosaminoglycans. All determinations were repeated one or more times and the molecular weights calculated for the 18%, 40% and 50% alcohol fractions were 94,000, 82,000 and 43,500 respectively. These molecular weights were inconsistent with the behaviour of the glycosaminoglycans on gel chromatography on Sephadex G-200 (Fig. 9).

The end group estimation technique described by Robinson and Hopwood (1973) relies upon the efficient alkaline cleavage of the xylosyl-serine linkage. In 0.5 M KOH, the xylosyl-serine bond is cleaved by a β-elimination reaction in which the serine is converted to dehydroalanine (Anderson et al, 1965). If this reaction is carried out in the presence of NaB^3H_4 the xylose at the reducing terminal of each chain is reduced to ^3H-xylitol (Fig. 10). Robinson and Hopwood (1973) showed, using cartilage proteoglycan, that the β-elimination reaction was complete after 4 days at 4° C and that there was no evidence of further degradation of the linkage region by peeling reactions (Lloyd et al, 1966) or of random chain cleavage.

In β-elimination reactions of this type a proton is released from the α-carbon of the serine and a nucleophile (xylose at the reducing end of the glycosaminoglycan chain) from the β-carbon resulting in the formation of a double bond (Fig. 10). Masking of the amino and carboxyl groups in serine is reportedly essential for efficient β-elimination. Electron withdrawing groups, such as those found in peptide linkage, facilitate elimination primarily by increasing the acidity of the α-hydrogen atom (Neuberger et al, 1966, Stern et al, 1971 and Inoue and Iwasaki, 1976). In view of the extensive proteolytic digestion used in the isolation of the ligament glycosaminoglycans, incomplete cleavage of the xylosyl-serine linkage was considered a likely explanation for

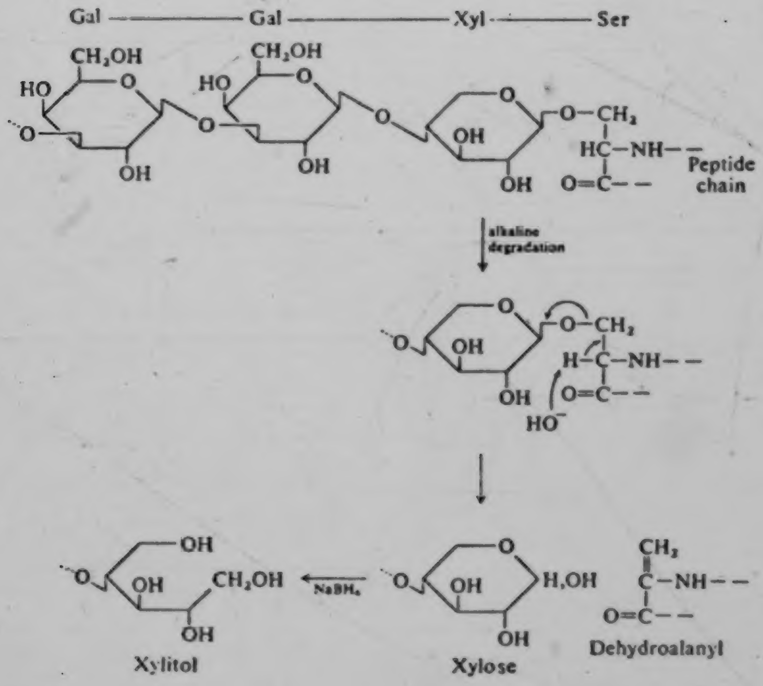

Gal
Gal
Xyl
Ser
CH₂OH
HO
O
OH
CH₂OH
HO
O
OH
O
OH
OH
O—CH₂
HC—NH— —
O=C— —
Peptide chain
alkaline degradation
O
OH
OH
O—CH₂
H—C—NH— —
O=C— —
HO⁻
OH
OH
CH₂OH
OH
Xylitol
NaBH₄
O
OH
H,OH
OH
Xylose
CH₂
C—NH— —
O=C— —
Dehydroalanyl

Fig. 10 Alkaline β Elimination and $NaBH_4$ Reduction of the Linkage Region of the Galactosaminoglycans

the high molecular weights determined. This was confirmed by amino acid analyses of the alkaline borohydride treated glycosaminoglycans. The uronic acid to serine ratio was still 70 - 80% of that found with the untreated material, showing that few xylosyl-serine linkages had been cleaved.

In view of these problems and the fact that the reductive end-labelling of chondroitin sulphate from cartilage proteoglycan was consistent with published reports, proteoglycans isolated from the periodontal ligament and from bovine skin, as well as that from bovine nasal cartilage, were used as substrates for alkaline cleavage and borohydride reduction. The proteoglycans from bovine periodontal ligament and bovine skin were obtained by sequential extraction with 0.1 M NaCl, 2 M NaCl (only used with periodontal ligament) and 4 M guanidinium chloride. They were purified by DEAE-cellulose chromatography in 7 M urea, cesium chloride density gradient centrifugation and gel chromatography on Sepharose 2B or 6B. See section 2.2.6 for the preparation and purification and section 4.3 for the characteristics of these proteoglycans. The reductively end-labelled glycosaminoglycans obtained from these purified proteoglycan preparations were fractionated by CPC and alcohol precipitation and subjected to gel chromatography on Sephadex G-200 (Fig. 11). For all fractions (except the 18% ethanol fraction obtained from 4 M guanidinium chloride extracted, ligament, proteoglycan) the peaks of radioactivity and hexuronic acid - positive material were displaced by several fractions and calculation of the number average molecular weight of each fraction showed that fractionation of the glycosaminoglycans had occurred on the basis of molecular size. The number average molecular weights of the 18, 40 and 50 percent alcohol fractions of ligament glycosaminoglycans were 3.08×10^4, 2.98×10^4 and 2.10×10^4 respectively. The glycosaminoglycans isolated from skin and cartilage proteoglycans (25 and 40% ethanol fractions respectively) gave number average molecular weights of 1.82×10^4 and 2.10×10^4 respectively and all molecular weights calculated were consistent with the behaviour of the glycosaminoglycans on Sephadex G-200.

The application of this type of end-group analysis to the

Fig. 11. Gel Chromatography on Sephadex G-200 of End-Labelled Glycosaminoglycan Fractions Obtained from Proteoglycans by Alkaline Cleavage and Reduction with Tritiated Borohydride

Proteoglycan samples from periodontal ligament bovine nasal septum and bovine skin were isolated and purified as described in section 2.2.6. These samples (approximately 250 µg as uronic acid) were digested with 0.5 M KOH and reductively labelled with tritiated sodium borohydride. Excess borohydride was destroyed, the samples desalted on Sephadex G-25 in pyridine acetate buffer, lyophilized and then fractionated using CPC and alcohol precipitation. End-labelled glycosaminoglycan samples were then applied to a G-200 column (1 x 90 cm) equilibrated with 0.2 M NaCl buffered with 0.02 M imidazole, pH 6.0. The column was eluted with this buffer at a rate of 4 ml/hr. and the 2 ml fractions collected were analysed for uronic acid and radioactivity.

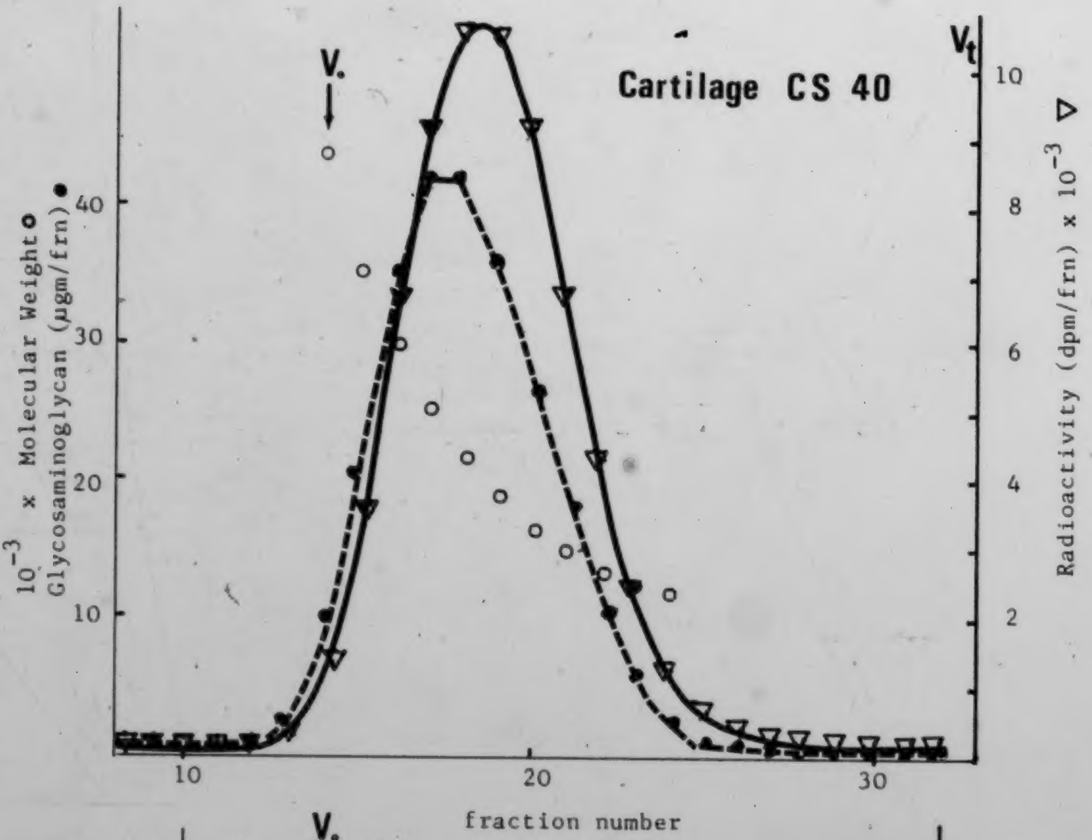
Cartilage CS refers to the end-labelled chondroitin sulphate obtained from bovine nasal cartilage proteoglycan and precipitated from 40% ethanol.

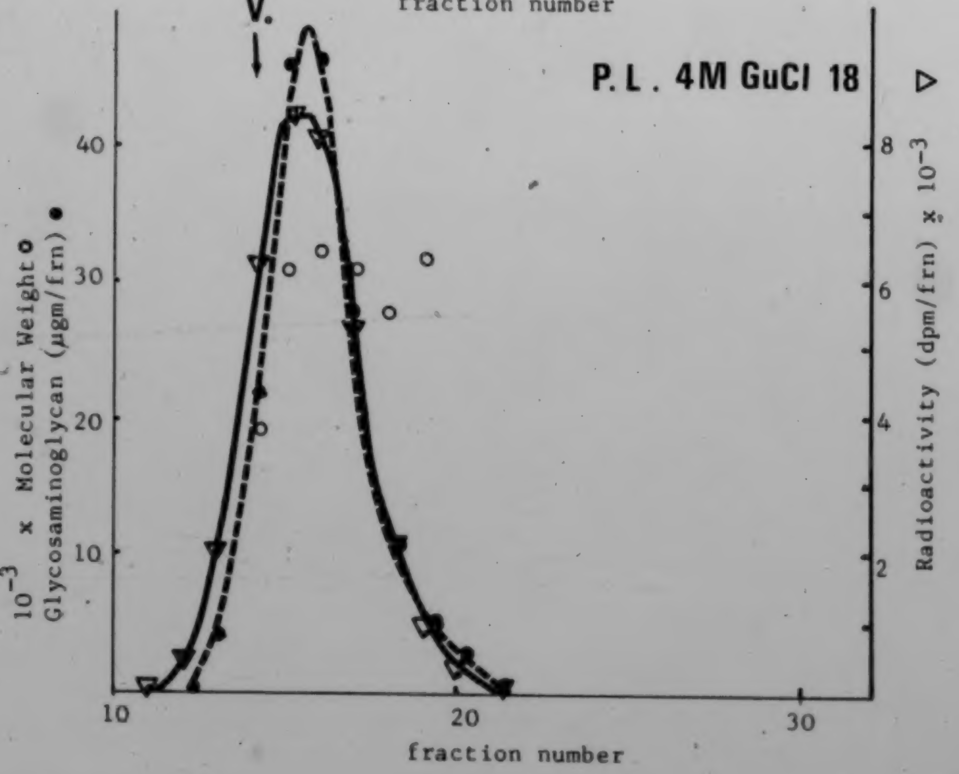
PL 4 M GuCl 18 refers to the end-labelled glycosaminoglycan obtained from periodontal ligament proteoglycan (4 M guanidinium chloride extracted) and precipitated from 18% ethanol.

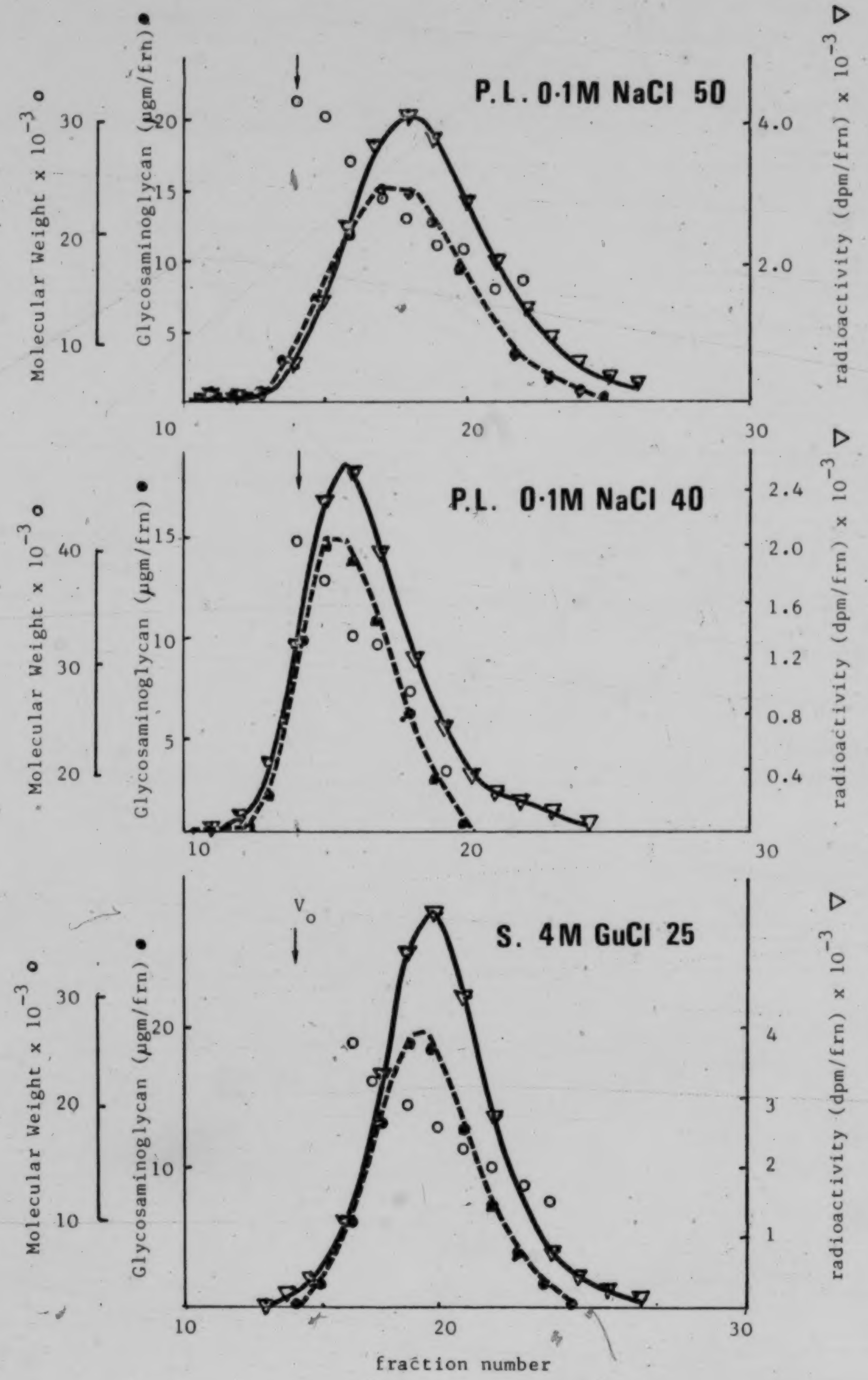
PL 0.1 M NaCl 50 refers to the end-labelled glycosaminoglycan obtained from periodontal ligament proteoglycan (0.1 M NaCl extracted) and precipitated from 50% ethanol.

PL 0.1 M NaCl 40 refers to the end-labelled glycosaminoglycan obtained from periodontal ligament proteoglycan (0.1 M NaCl extracted) and precipitated from 40% ethanol.

S 4 M GuCl 25 refers to the end-labelled glycosaminoglycan obtained from skin proteoglycan (4 M guanidinium chloride extracted) and precipitated from 25% ethanol.


Cartilage CS 40
V.
V_t
10^{-3} x Molecular Weight ○
Glycosaminoglycan (μgm/frn) ●
Radioactivity (dpm/frn) x 10^{-3} ▷
10
20
30
40
2
4
6
8
10
fraction number


P. L . 4M GuCl 18
V.
10^{-3} x Molecular Weight ○
Glycosaminoglycan (μgm/frn) ●
Radioactivity (dpm/frn) x 10^{-3} ▷
10
20
30
40
2
4
6
8
fraction number


P.L. 0·1M NaCl 50
P.L. 0·1M NaCl 40
S. 4M GuCl 25
Molecular Weight x 10^{-3} ○
Glycosaminoglycan (μgm/frn) ●
radioactivity (dpm/frn) x 10^{-3} ▽
V_o
10
20
30
5
15
40
2.0
4.0
0.4
0.8
1.2
1.6
2.4
1
2
3
4
fraction number

determination of the molecular weight of the galactosaminoglycans of the skin and periodontal ligament requires some knowledge of the carbohydrate-protein linkage. Stern et al (1971) showed that the dermatan sulphate - protein linkage in pig skin was the same as that found for chondroitin 4-sulphate (Lindahl and Rodén, 1966 and Rodén and Smith, 1966), chondroitin 6-sulphate (Helting and Rodén, 1968) and heparin (Lindahl, 1966), shown in Fig. 10. Furthermore they showed that over 90% of the xylosyl-serine linkages in a dermatan sulphate peptide, isolated by trypsin digestion, could be cleaved by treatment with 0.4 M KOH. Thus the carbohydrate-protein linkage in bovine periodontal ligament and skin proteoglycans may be confidently expected to be of the same type. Moreover cleavage of the carbohydrate-protein linkage would be expected to be as efficient, or probably more efficient, than with the dermatan sulphate peptides isolated by Stern et al (1971).

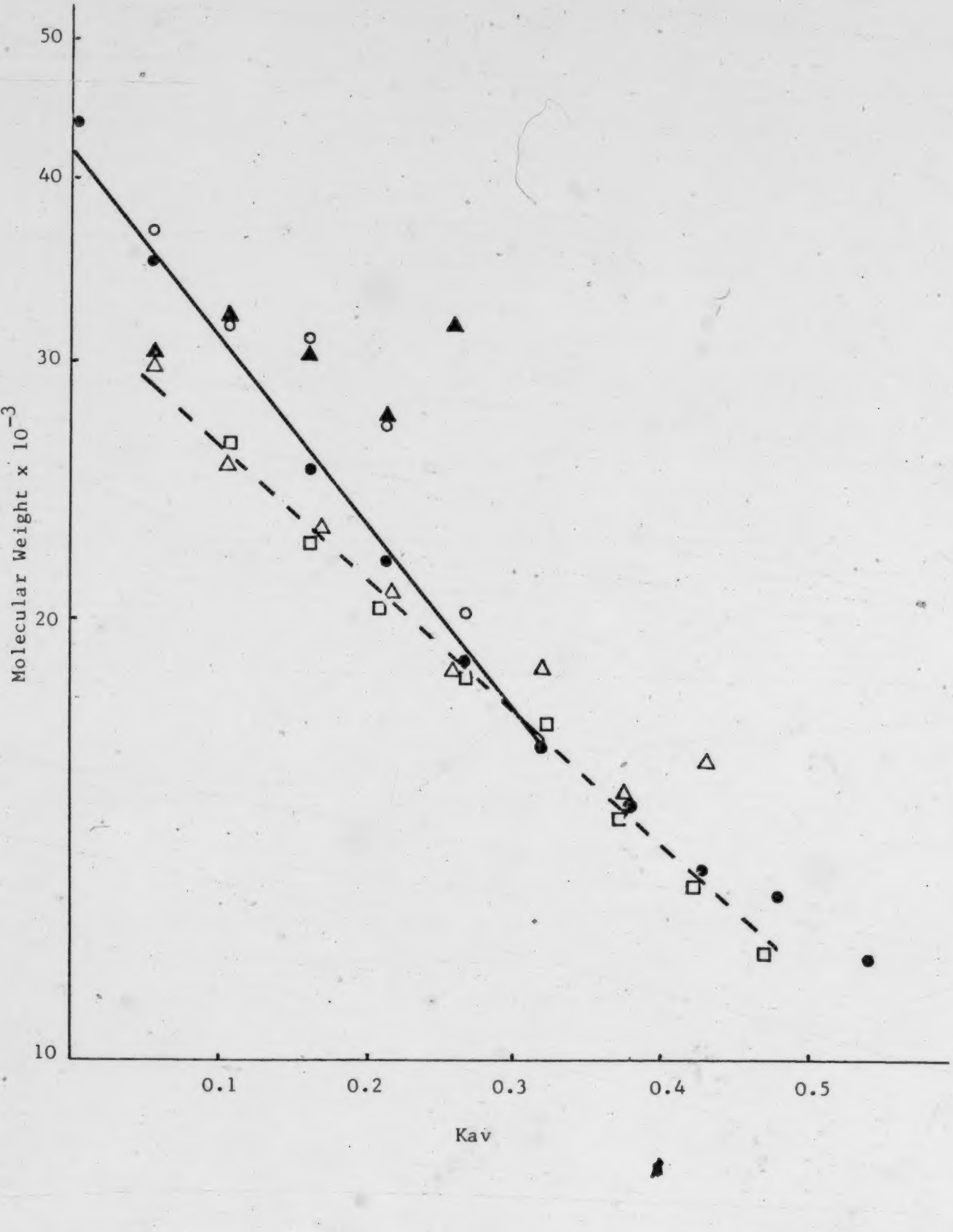
Inaccuracy in the determination of molecular weights by this technique of end group analysis can also be introduced by errors in the determination of the uronic acid content of the respective glycosaminoglycans. The uronic acid content was determined on glycosaminoglycans isolated from the whole ligaments whereas molecular weights were determined on glycosaminoglycans isolated from the purified proteoglycans. However the equivalent fractions should be expected to have very similar composition. The uronic acid content of the skin glycosaminoglycans was not determined due to the limited material available and these fractions were assumed to have a similar composition to the ligament glycosaminoglycans. Moreover since uronic acid contents of the glycosaminoglycan samples varied by only a few percent, the errors introduced in this determination would be expected to be small. Error could also arise in the determination of the specific activity of the NaB^3H_4. The chondroitin sulphate liberated by alkaline cleavage of the chondroitin sulphate proteoglycan and the other glycosaminoglycans liberated by alkaline cleavage of their respective proteoglycans should be effectively identical substrates for $NaBH_4$ reduction. Thus any errors in molecular weight determination arising from the standardization of the NaB^3H_4 should only reflect errors in the determination of the chondroitin sulphate molecular weight. This was determined from equilibrium sedimentation and the results obtained ($\bar{M}n$ 21,700, $\bar{M}w$ 24,400) were very similar to those previously reported (Luscombe and Phelps, 1967, Wasteson, 1969 and Robinson and Hopwood, 1973).

Since Sephadex G-200 fractionation of end-labelled glycosaminoglycans conveniently yields a large number of essentially monodisperse fractions, each with a different molecular size, weight average ($\bar{M}w$) as well as number average ($\bar{M}n$) molecular weights can be calculated (Table 11). A convenient measure of the molecular weight distribution of a polymer may be obtained from $\bar{M}w/\bar{M}n$ ratios (Flory, 1953). A monodisperse preparation has a $\bar{M}w/\bar{M}n$ ratio of 1.00. The $\bar{M}w/\bar{M}n$ ratio obtained for chondroitin sulphate prepared from bovine nasal cartilage proteoglycan was 1.13 and is closely comparable with the results obtained by Hopwood and Robinson (1973). The ratio for the glycosaminoglycans isolated from the periodontal ligament were all lower (1.10 and 1.04 for the 40 and 50% alcohol fractions respectively), suggestive of less polydispersity. Moreover the 18% alcohol fraction, obtained from the 4 M guanidinium chloride extracted ligament proteoglycan, gave an $\bar{M}w/\bar{M}n$ ratio of 1.004, indicative of a largely monodisperse preparation. This result was consistent with the very sharp peak obtained on Sephadex G-200 chromatography. However the calculation did not include the low molecular weight fraction obtained at the void volume (Fig. 11, PL 4 M GuCl 18).

In Fig. 12 the partition coefficient Kav (Laurent and Killander, 1964) between Sephadex G-200 and buffer, calculated for the glycosaminoglycan fractions obtained from periodontal ligament, skin and cartilage proteoglycans, is plotted against the calculated molecular weight of each polysaccharide sample. Some differences are observed between the various glycosaminoglycan fractions. The skin 25% ethanol fraction and the ligament 50% ethanol fraction which show similar elution behaviour tend to differ slightly from the ligament 40% ethanol fraction and the cartilage 40% ethanol fraction which resemble each other in elution behaviour. The differences in slope of the lines produced may be due to different rates of diffusion, however the general similarity of the plots of the various glycosaminoglycan fractions suggests that they have very similar distribution on gel filtration and hence similar conformation. Although the plot of the 18% alcohol fraction is markedly different from those of the other glycosaminoglycan fractions, the average molecular weight ($\bar{M}n = 30.8 \times 10^3$) plotted at the peak height (Kav 0.08) fits the same straight line relationship. A monodisperse fraction

Fig. 12 Fractionation Coefficient (Kav; Laurent and Killander, 1964) between Sephadex G-200 and Buffer for a Series of Glycosaminoglycan Fractions Derived from Bovine Nasal Cartilage Proteoglycan, Periodontal Ligament Proteoglycans and Bovine Skin Proteoglycan as a Function of Molecular Weight

The partition coefficient (Kav) between Sephadex G-200 and buffer calculated for glycosaminoglycan fractions shown in the previous figure is plotted against the molecular weight of each glycosaminoglycan sample. The 40% ethanol fraction from bovine nasal cartilage proteoglycan (●), 18% ethanol fraction from 4 M guanidinium chloride extracted, periodontal ligament proteoglycan (▲), 40% ethanol fraction (○) and 50% ethanol fraction (△) from 0.1 M NaCl extracted, periodontal ligament, proteoglycan and 25% ethanol fraction from bovine skin proteoglycan (□) are shown. For the sake of clarity lines for the 40% ethanol fraction from bovine nasal cartilage proteoglycan (-●-) and the 25% ethanol fraction from bovine skin proteoglycan (-□-) only are drawn.


Molecular Weight x 10^{-3}
50
40
30
20
10
0.1
0.2
0.3
0.4
0.5
Kav

Fraction		$\bar{M}n \times 10^{-3}$	$\bar{M}w \times 10^{-3}$	$\frac{\bar{M}w}{Mn}$
Periodontal ligament				
4 M GuCl	18	30.8	30.9	1.004
0.1 M NaCl	40	29.8	33.0	1.10
	50	21.0	21.8	1.04
Skin	25	18.2	19.2	1.05
Cartilage	40	21.4	23.6	1.127

Table 11. Molecular Weight Values for Bovine, Periodontal Ligament, Skin and Cartilage Glycosaminoglycans

Number average ($\bar{M}n$) and weight average ($\bar{M}w$) molecular weights were calculated after alkaline elimination and $\mathrm{NaB^3H_4}$ reduction of purified bovine skin, cartilage and periodontal ligament proteoglycans. The isolated glycosaminoglycan samples were chromatographed on Sephadex G-200 (Fig. 11) and the molecular weights calculated from the results obtained.

would be expected to produce a horizontal line such as that produced by the 18% ethanol fraction. Deviation of the lower molecular weight cartilage chondroitin sulphate fractions from a straight line plot and the scatter observed with some fractions are probably due to errors introduced by the analysis of small amounts of material, particularly in the lower regions of the chromatograph peaks.

3.4.4 The Copolymeric Nature of Ligament Glycosaminoglycans

From the iduronic acid analysis of the 18%, 25%, 40% and 50% ethanol fractions it appeared that these ligament glycosaminoglycan fractions contained both glucuronic and iduronic acid in varying proportions - that is they appear to be hybrid structures. Refractionation of the 40% ethanol fraction at 30%, 40% and 50% ethanol concentrations gave no precipitation at 30% ethanol suggesting that the iduronic acid observed in this fraction was not due to contamination with a glycosaminoglycan rich in iduronic acid. The profiles on cellulose acetate strips were also characteristic of this type of copolymer (Fransson and Roden, 1967a, Habuchi et al, 1973 and Inoue and Iwasaki, 1976) (see Fig. 8). However more definitive evidence for the copolymeric nature of these glycosaminoglycans was provided by hyaluronidase digestion and also by periodate oxidation followed by alkali cleavage.

Under controlled conditions of pH and temperature, unsubstituted L-iduronosyl residues can be oxidized with periodate, while D-glucuronosyl residues are largely unaffected (Fransson, 1974). Oxidation can be demonstrated by cleavage of the oxidized residues with alkali (Fransson and Carlstedt, 1974). In order to establish conditions under which minimal oxidation of chondroitin sulphate occurred, samples of chondroitin sulphate were oxidized at pH 3.0, pH 2.5 and pH 2.0 and cleaved in alkali. The results of this experiment are shown in Fig. 13. As can be seen, the degree of oxidation at pH 2.0 is very small, whereas oxidation at pH 3.0, reported to be negligible (Fransson, 1974), was quite extensive in my hands. Thus oxidation of ligament glycosaminoglycan samples was routinely carried out at pH 2.0.

Hyaluronidase has been shown to cleave β-hexosaminic bonds to glucuronic acid but not to those involving iduronic acid. Thus degradation with hyaluronidase is considered indicative of the presence of glucuronic acid in these polymers.

The results of hyaluronidase digestion and periodate oxidation - alkali cleavage indicate that all four glycosaminoglycan fractions contain copolymers and give some idea of their uronic acid distribution. Since they appear to belong to separate families of glycosaminoglycan, results of digestion of the 40 and 50% alcohol fractions and 18 and 25% alcohol fractions will be discussed separately.

The extent of periodate oxidation and alkali cleavage of the 40 and 50% alcohol fractions is consistent with the premise that the iduronic acid and glucuronic acid in these fractions are contained within the same glycosaminoglycan chains, i.e. they are copolymers. The degradation of the 50% alcohol fraction is only slightly greater than the control at pH 2.0 (Fig. 14 and 13) suggesting that the small proportion of iduronic acid is either at terminal regions of the chain or that residues within the chain are not susceptible to periodate oxidation. Extensive degradation of the 40% alcohol fraction is observed on Sephadex G-200 (Fig. 14), indicative of a number of

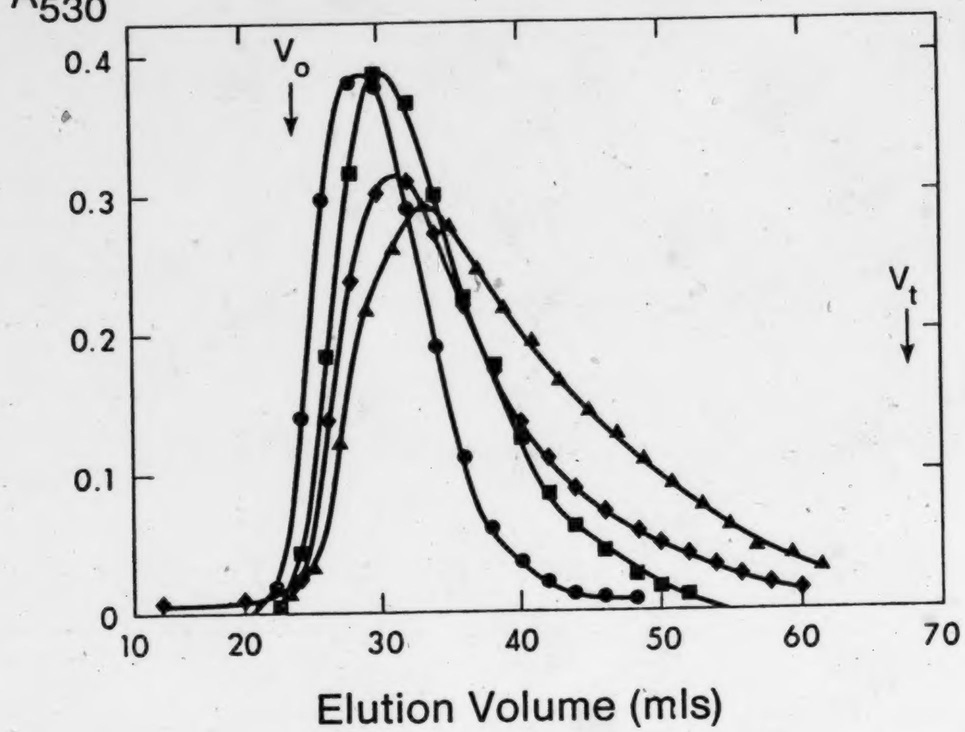
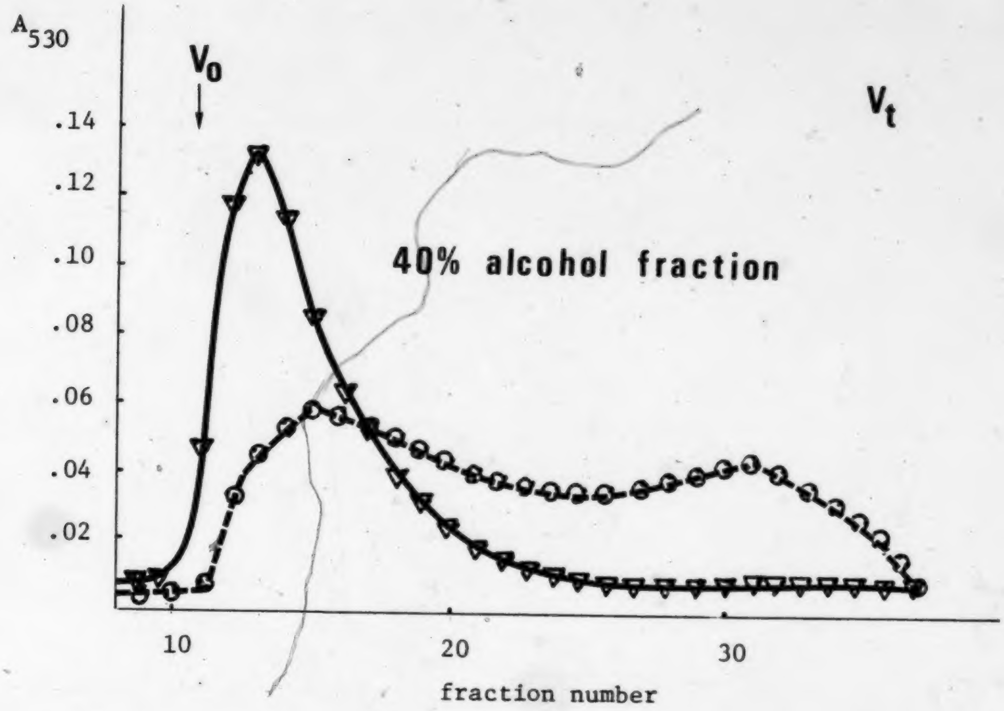


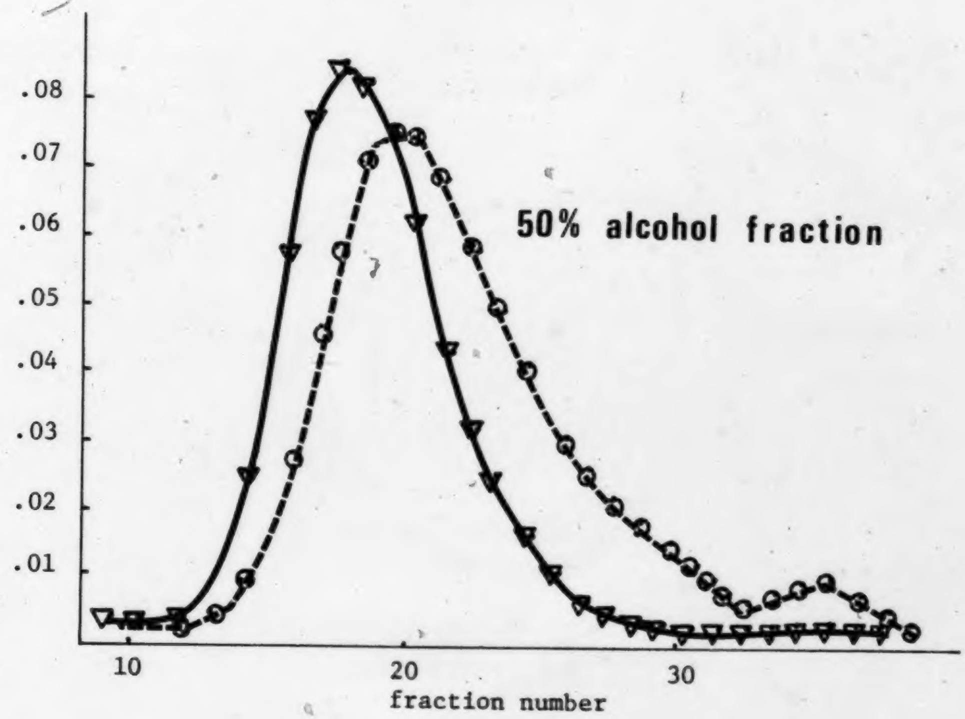
Fig. 13 Effect of pH on the Periodate Oxidation and Alkaline Elimination of Chondroitin Sulphate

2.0 mg samples of standard chondroitin 4-sulphate were oxidized in 20 mM sodium metaperiodate containing 50 mM sodium citrate and incubated in the dark at 4° C for 24 hrs. at pH 2.0 (■), 2.5 (◆), or 3.0 (▲). The reaction was terminated by the addition of 0.1 volumes of 10% aqueous D-mannitol. After treatment with alkali (pH 12.0 for 30 mins.), the reaction mixture was neutralized, applied to a Sephadex G-200 column (1 x 90 cm) and eluted with pyridine-acetic acid buffer (0.2 M in acetic acid) at pH 5.0. 2 ml samples were analysed for uronic acid. V_o and V_t are the elution volumes of blue dextran and ^3H_2O respectively. (●) untreated chondroitin 4-sulphate.

Fig. 14. Gel Chromatography on Sephadex G-200 of the 40 and 50% Alcohol Fractions after Periodate Oxidation and Alkali Elimination

The 40% and 50% alcohol fractions of ligament glycosaminoglycans were oxidized with 20 mM periodate at pH 2.0, in the dark, at 4° C for 24 hrs. After alkali cleavage and neutralization, the reaction mixture was applied to a Sephadex G-200 column (1 x 90 cm) and eluted with pyridine-acetic acid buffer (0.2 M in acetic acid). (▽) untreated glycosaminoglycan, (o) oxidized alkali cleaved sample, Vo and Vt are the elution volumes of blue dextran and ^3H_2O respectively


A_{530}
V_0
V_t
.14
.12
.10
.08
.06
.04
.02
40% alcohol fraction
10
20
30
fraction number


.08
.07
.06
.05
.04
.03
.02
.01
50% alcohol fraction
10
20
30
fraction number

periodate susceptible iduronic acid residues within the chain. However the products of periodate oxidation are almost completely excluded from Sephadex G-50 (Fig. 15), suggesting that the iduronic acid residues are separated by longer polymer regions that contain no periodate susceptible iduronic acid residues.

The copolymeric nature of these fractions is further supported by the pattern of hyaluronidase digestion and the analyses of digestion products. Hyaluronidase digestion degrades both fractions to tetra, hexa and octasaccharides together with a small proportion of larger oligosaccharides. The proportion of larger oligosaccharides is greater in the digest of the 40% alcohol fraction consistent with the higher iduronic acid content and hence fewer hyaluronidase susceptible linkages in these chains (Fig. 15). Approximately 50% and 30% of the total iduronic acid in the digests of the 40 and 50% alcohol fractions respectively, is found in oligosaccharides that are retarded on Sephadex G-50 (Table 12). These products of hyaluronidase digestion could only have arisen from single chains containing both iduronic and glucuronic acid.

A large proportion of the iduronic acid in the 40 and 50% ethanol fractions occurs in regions that are not susceptible to hyaluronidase (frns. 1, Fig. 15 and Table 12). These regions do, however, contain some glucuronic acid (approximately 40% of the total uronic acid in the frns.). This would suggest that the majority of these glucuronic acid residues occur as single units within blocks of iduronic acid containing polymer, since single glucuronic acid - N-acetylgalactosamine sulphate periods do not constitute hyaluronidase-susceptible sites (Ludowieg et al, 1961). The apparent absence of a block region containing only iduronic acid-N-acetylgalactosamine sulphate residues but a region of interspersed iduronic acid and glucuronic acid - N-acetylgalactosamine sulphate residues may explain the absence of small oligosaccharide degradation products that would be expected to arise from the periodate oxidation - alkali cleavage of such block regions.

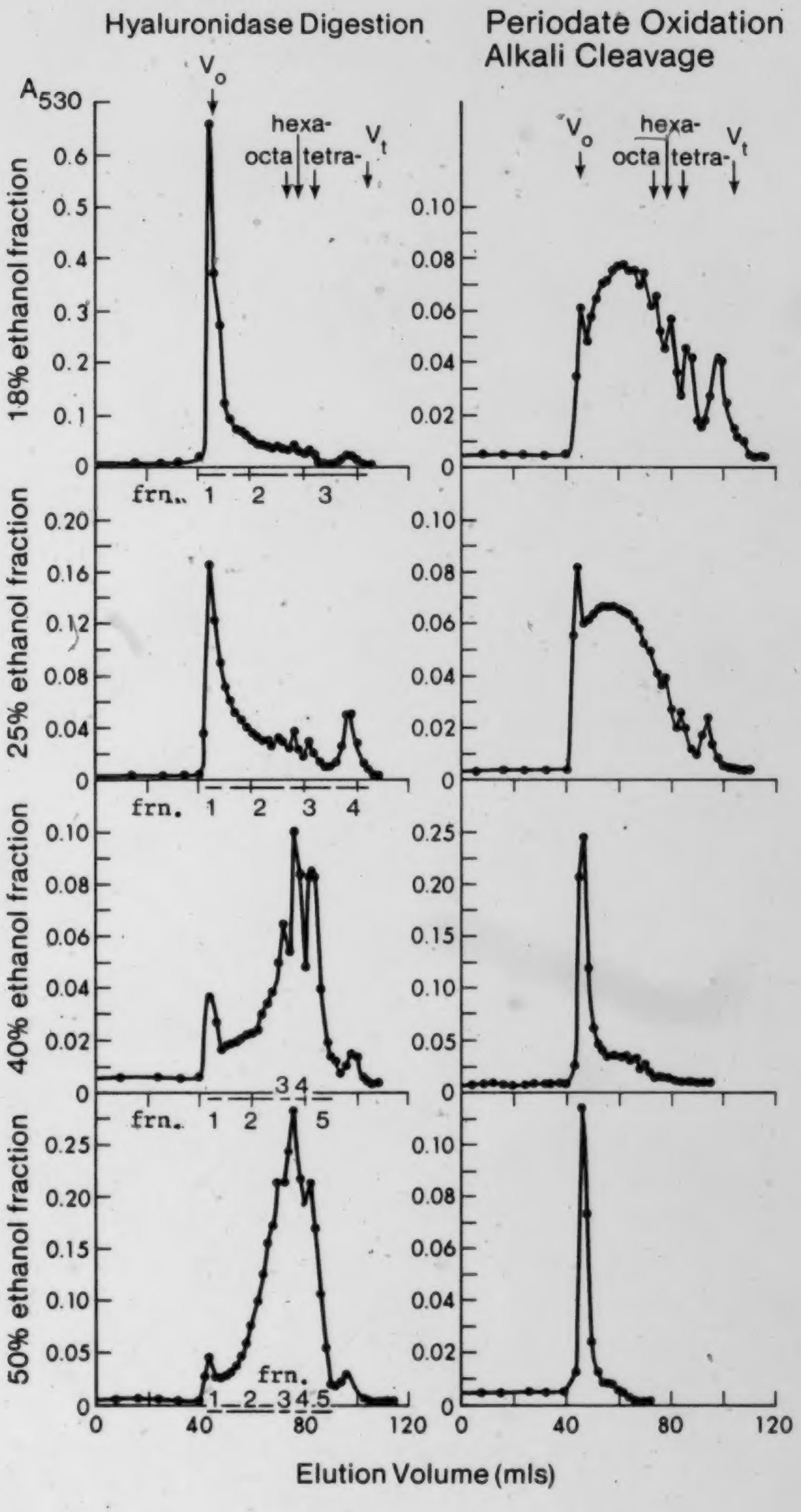
Most (approximately 70% of the total) of the glucuronic acid residues in the 40 and 50% alcohol fraction occur in large block regions that are cleaved by hyaluronidase to tetra, hexa and octasaccharides and are resistant to controlled periodate oxidation - alkali cleavage.

Fig. 15 Chromatography on Sephadex G-50 of the Periodate Oxidized - Alkali Cleaved and Hyaluronidase Digested, Ligament Glycosaminoglycan, Fractions

Alcohol fractions of ligament glycosaminoglycans were oxidized with 20 mM periodate at pH 2.0, in the dark for 24 hrs. After alkali cleavage and neutralization, the reaction mixture was applied to a Sephadex G-50 column (1 x 120 cm) and eluted with 0.2 M pyridine acetate (0.2 M in acetate), pH 5.0. 2 ml fractions were collected and analysed for uronic acid.

Alcohol fractions of ligament glycosaminoglycans were also digested with ovine testicular hyaluronidase (1,400 N.F. units/mg glycosaminoglycan) in 0.1 M sodium acetate, 0.1 M NaCl at pH 5.0 and 37° C for 18 hrs. The reaction was stopped by 2 min. incubation at 100° C and the digested mixture applied to the Sephadex G-50 column and eluted with pyridine acetate as described.

2 ml fractions were analysed for uronic acid. The frns. marked were pooled, lyophilized and analysed for uronic acid and dermatan sulphate. Octa, hexa and tetra refer to the elution positions of octa, hexa and tetrasaccharides obtained by hyaluronidase digestion of chondroitin sulphate. Vo and Vt are the elution positions of blue dextran and ^3H_2O respectively.


Hyaluronidase Digestion
Periodate Oxidation
Alkali Cleavage
A530
V0
hexa-
octa
tetra-
Vt
18% ethanol fraction
25% ethanol fraction
40% ethanol fraction
50% ethanol fraction
frn. 1 2 3
frn. 1 2 3 4
frn. 1 2 3 4 5
frn. 1 2 3 4 5
0
0.1
0.2
0.3
0.4
0.5
0.6
0.04
0.08
0.12
0.16
0.20
0.02
0.06
0.10
0.05
0.15
0.25
0.20
0 40 80 120
Elution Volume (mls)

The possibility exists that the 50% alcohol fraction is in fact a chondroitin sulphate contaminated with a small amount of the copolymeric material such as found in the 40% alcohol fraction. If this were the case, however, the proportion of iduronic acid retarded on Sephadex G-50 would be expected to be the same in the 40% and 50% alcohol fractions, which is not the case (Table 12).

These fractions, however, are probably not homogeneous but contain a range of glucuronic acid rich copolymers. Furthermore the occurence of a small proportion of chondroitin sulphate homopolymers (containing no iduronic acid) cannot be eliminated.

The copolymeric nature of the 18 and 25% alcohol fractions is indicated by the products of hyaluronidase digestion. Although extensive degradation of the 18% alcohol fraction is not obvious on Sephadex G-50 (Fig. 15) the degradation of both the 18 and 25% alcohol fractions is evident on Sephadex G-200 (Fig. 16). The extent of degradation by both hyaluronidase and periodate alkali was dependant on the iduronic acid content of the polymers and suggests that both are copolymers containing different proportions of glucuronic acid. The apparent aggregation observed with the hyaluronidase digest of the 25% alcohol fraction (Fig. 16) is unusual but may be of the type reported by Fransson (1976) occurring between copolymeric glycosaminoglycans, though this fraction contains little, if any, glucuronic acid. In both the 18 and 25% alcohol fractions the number of residues resisting periodate oxidation - alkali cleavage is greater than expected from the glucuronic acid content of the polymer. This is consistent with the results of Fransson (1974) and may be due to sulphation of the iduronic acid residue at C2 or C3 (thus rendering the residues periodate insensitive). There is also the possibility that a small proportion of the non-sulphated L-iduronic acid units had the $^1C_4$ conformation and thus diaxial glycol groupings at C2 and C3. This conformation (similar to that of glucuronic acid in chondroitin sulphate) renders the iduronic acid less sensitive to periodate oxidation.

The results in Table 12 show that nearly all the iduronic acid in the 18% alcohol fraction, occurs in long block regions that appear to be largely free of glucuronic acid (frn. 1, Fig. 15 and Table 12).

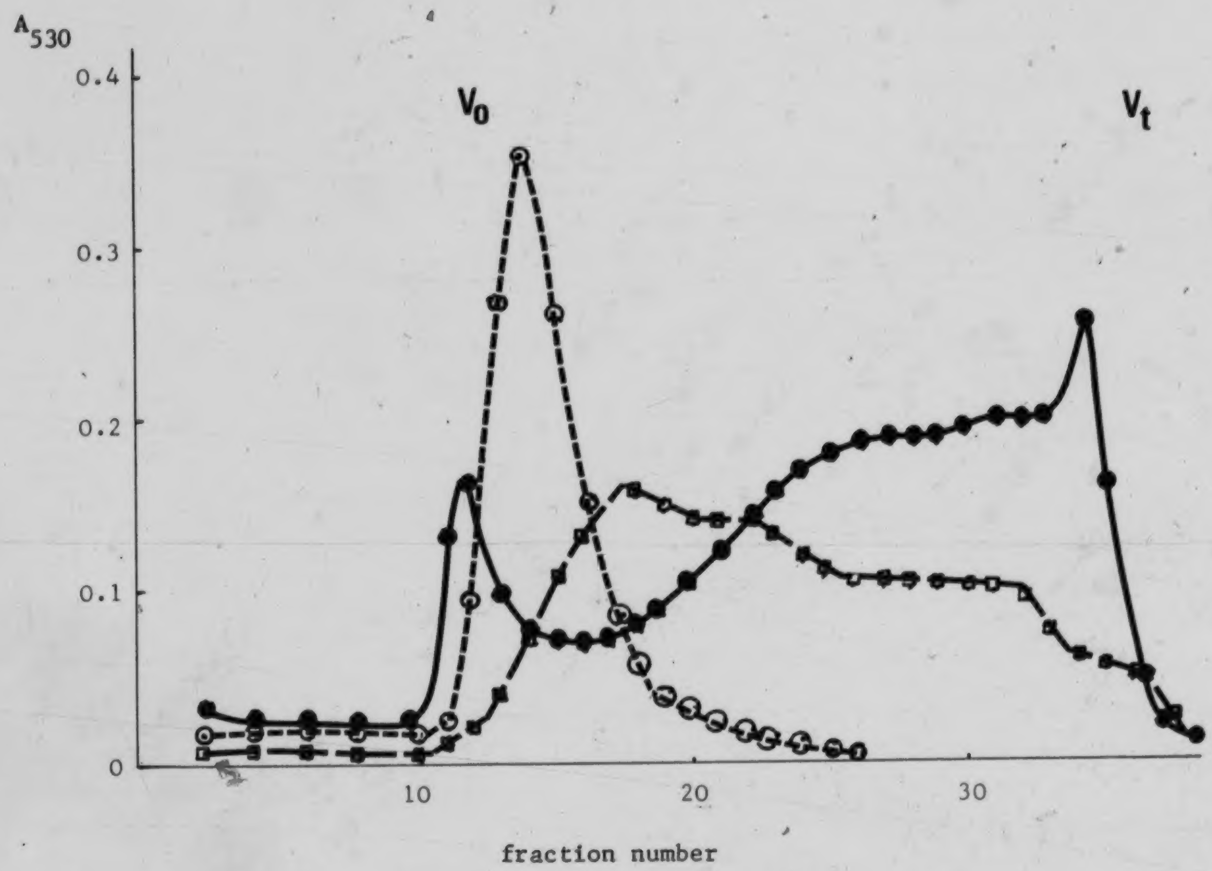


Fig. 16 Gel Chromatography of Hyaluronidase Digested 18% and 25% Alcohol Fractions of Periodontal Ligament Glycosaminoglycans on Sephadex G-200

The 18 and 25 percent ethanol fractions of periodontal ligament glycosaminoglycans were digested with hyaluronidase (1,432 N.F. units/mg glycosaminoglycan) in 0.1M sodium acetate, 0.1M NaCl at pH 5.0 and 37°C for 18 hrs. After 10 min. incubation at 100°C the digested material was applied to a Sephadex G-200 column (90 x 1 cm) and eluted with 0.2M pyridine acetate pH 5.0. 2 ml fractions were collected and analysed for uronic acid. V_o and V_t are the elution volumes of blue dextran and ^3H_2O respectively. (⊙) 18% ethanol fraction prior to hyaluronidase digestion. (▣ and ●) hyaluronidase digested 18 and 25 percent ethanol fractions respectively.

alcohol fraction	frn.	% total uronic acid	% total iduronic acid	iduronic acid content of peak %
18%	1	68.1	88.9	99.0
	2	15.5	9.1	48.9
	3	16.5	2.0	10.7
25%	1	27.0	45.2	100
	2	45.8	48.2	69.0
	3	10.3	4*	25*
	4	16.9	3*	8*
40%	1	8.2	35.5	61.1
	2	14.3	43.5	44.2
	3	19.9	8.7	6.7
	4	29.6	6*	3*
	5	28.1	6*	3*
50%	1	4.1	52.3	61.2
	2	31.8	28.1	4.6
	3	15.2	3*	1*
	4	27.8	3*	1*
	5	21.0	4*	1*

Table 12. Analysis of the Frns, Obtained from Sephadex G-50 Chromatography of Hyaluronidase Digested Ligament Glycosaminoglycan Fractions

The frns. indicated in Fig. 15 were collected, lyophilized and analysed for uronic acid by the carbazole technique and for iduronic acid by the Di Ferrante technique. % total uronic acid and % total iduronic acid refer to the amount of uronic acid and iduronic acid found in the designated frns., expressed as a percentage of the total uronic acid and a percentage of the total iduronic acid recovered from the column. Iduronic acid content of frn. refers to the percentage of iduronic acid to the total uronic acid in that frn.. * indicates figures calculated from iduronic acid estimations very close to the limits of detection.

Similarly the glucuronic acid also appears to occur in block regions since after hyaluronidase digestion it is nearly all found in small oligosaccharides (frns. 2 and 3, Fig. 15 and Table 12). Hyaluronidase digestion of the 25% alcohol fraction suggests that this glycosaminoglycan contains large regions where iduronic and glucuronic acid occur interspersed together. Almost 50% of the digest is composed of oligosaccharides (frn. 2) that have an overall glucuronic : iduronic acid ratio of almost 1 to 2. The remainder of the polymer appears to be composed of block regions containing either iduronic acid, giving rise to frn. 1, or glucuronic acid, giving rise to frns. 3 and 4, on hyaluronidase digestion.

Thus, even though it seems valid to pool these glycosaminoglycan fractions into polymers containing predominantly glucuronic acid and polymers containing predominantly iduronic acid, the four fractions appear to be distinct hybrid glycosaminoglycan species which differ in the proportion and distribution of glucuronic and iduronic acid, in the position of sulphation and in molecular size.

CHAPTER 4

THE PROTEOGLYCANS OF THE PERIODONTAL LIGAMENT

4.1 INTRODUCTION

There is now general agreement that most glycosaminoglycans occur in the native state covalently bound to protein as proteoglycans (see section 1.3 for a discussion of proteoglycan structure), which are distinguished by the type of glycosaminoglycan chain and by the protein core. The most intensely studied proteoglycan is that from cartilage. Investigation of the structure of this type of proteoglycan was greatly facilitated by the development of a new method of extraction and purification using 4 M guanidinium chloride extraction and purification by density gradient centrifugation, in the presence of guanidinium chloride (Hascall and Sajdera, 1969). In contrast to cartilage, other connective tissues such as skin or periodontal ligament contain much smaller amounts of proteoglycan and the guanidinium chloride extracts contain much larger amounts of non-proteoglycan proteins. These factors and the fact that proteoglycans from these tissues have lower buoyant densities, which make them difficult to purify by CsCl density gradient centrifugation, are the major reasons for the limited study of this type of proteoglycan. Antonopoulos et al (1974) however, recently introduced a technique using DEAE-cellulose chromatography in 7 M urea, which overcame most of these problems and greatly facilitated the isolation and purification of non-cartilaginous proteoglycans. These techniques formed the basis of those used to extract and characterize the proteoglycans of the periodontal ligament.

It has become increasingly apparent that the presence of proteolytic enzymes within connective tissues can cause partial degradation of the proteoglycans during extraction (Oegema et al, 1975a, Pearson and Mason, 1977). For this reason extractions were carried out at neutral pH to minimize the effect of cathepsin D, an apparently ubiquitous connective tissue, acid protease (Barrett, 1975), and extraction solutions contained a number of protease inhibitors (see section 2.2.6.2.1).

Since very limited quantities of periodontal ligament could be obtained, extraction and purification techniques were first investigated using bovine skin. Moreover, preparation of the skin and also cartilage proteoglycans enabled valuable comparison with proteoglycans isolated from the periodontal ligament.

4.2 EXTRACTION

4.2.1 Extraction of Bovine Skin with Guanidinium Chloride Solutions

Bovine skin was extracted by three sequential treatments with 0.4 M guanidinium chloride followed by a similar treatment with 4 M guanidinium chloride. The yield of glycosaminoglycans, protein and collagen was determined as described in Table 13. The extraction of 90 percent of the dermatan sulphate was unexpected since, at the time these results were obtained, the extremely firm attachment of dermatan sulphate proteoglycan to the collagen matrix and hence low yield of dermatan sulphate proteoglycans was considered to be characteristic of this type of proteoglycan (Meyer, 1966, Toole and Lowther, 1966, Lowther et al, 1967). For the same reasons extraction of almost 50 percent of the dermatan sulphate in 0.4 M guanidinium chloride was even more unexpected, although Pearson et al (1975) in a preliminary study of the glycosaminoglycans of the periodontal ligament had reported that a small amount of dermatan sulphate could be extracted with 0.14 M NaCl.

4.2.2 Sequential Extraction of the Periodontal Ligament

In the hope of selectively extracting hyaluronic acid and the chondroitin sulphate and dermatan sulphate proteoglycans, periodontal ligament was extracted with 0.1 M NaCl (rather than 0.4 M guanidinium chloride), 2.0 M NaCl and 4 M guanidinium chloride. As can be seen from Table 14, 94% of the total uronic acid and 85% of the total dermatan sulphate was extracted from the ligament. Some selective extraction was apparent from the dermatan sulphate analyses as well as cellulose acetate electrophoresis of the isolated glycosaminoglycans. However almost 20% of the dermatan sulphate was extracted with 0.1 M NaCl.

Extract	% total HA	% total CS	% total DS	mg Protein	mg hyp
0.4 M GuCl					
extract 1	36.1	26.4	21.2	672.6	12.5
extract 2	40.6	9.7	11.2	213.2	10.5
extract 3	13.6	13.6	10.8	99.0	8.4
extract 1, 2 & 3	90.3	49.7	43.2		
4 M GuCl					
extract 4	5.1	21.3	21.7	780	73.4
extract 5	2.5	20.6	11.3	340	37.5
extract 6	1.9	5.8	14.6	158	23.5
extract 4, 5 & 6	9.5	47.7	47.6		
Residue	0.1	2.5	9.2		

Table 13. Yield of Proteoglycans(s) from Bovine Skin After Sequential Guanidinium Chloride Extraction

40 gm (dry weight) of milled bovine skin was extracted with 3 treatments of 0.4 M guanidinium chloride followed by 3 treatments 4.0 M guanidinium chloride as described in section 2.2.6.2. Samples of the extracts were dialysed against distilled water. Aliquots were analysed for protein (Lowry et al, 1951) and the remainder digested with papain. Samples of this mixture were analysed for hydroxyproline (Stegeman and Stalder, 1967). Hyaluronic acid (HA) and the sulphated glycosaminoglycans were separated by CPC precipitation. Chondroitin sulphate (CS) and dermatan sulphate (DS) were analysed by hyaluronidase digestion.

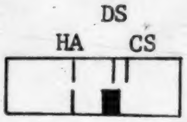
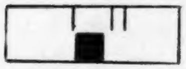
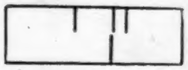
Extract	Uronic Acid		Dermatan Sulphate		Hyp	Cellulose Acetate Electrophoresis
	mg	% total	mg	% total	mg	
0.1 M NaCl 1	20.2	51.6	4.75	14.7	16.8	
0.1 M NaCl 2	1.55	4.0	0.44	1.4	2.83	
0.1 M NaCl 3	0.36	0.9	0.35	1.1	0.876	
Total 0.1 M NaCl	22.1	56.5	5.54	17.1	20.5	
2 M NaCl 1	3.10	7.9	*		0.54	
2 M NaCl 2	0.38	1.0	*		*	
2 M NaCl 3	0.38	1.0	*		*	
Total 2 M NaCl	3.86	9.9	*		0.54	
4 M GuCl 1	7.50	19.2	19.8	61.2	10.2	
4 M GuCl 2	2.68	6.9	2.07	6.4	5.87	
4 M GuCl 3	0.438	1.1	0.137	0.4	6.55	
Total 4 M GuCl	10.62	27.2	22.0	68.0	22.6	
Residue	2.53	6.5	4.8	14.8		

Table 14. Yield of Proteoglycans and Collagen From Bovine Periodontal Ligament

86 gm (wet weight)• of milled bovine periodontal ligament was extracted with three sequential treatments of each of the following solutions: 0.1 M NaCl, 2 M NaCl and 4 M guanidinium chloride (GuCl). All extractions were carried out at 4° C and the solutions contained the buffered protease inhibitor cocktail described in section 2.2.6.2.1 . Uronic acid was analysed by the carbazole technique (Bitter and Muir, 1962) after papain digestion and CPC precipitation of the glycosaminoglycans. Dermatan sulphate analyses of the glycosaminoglycans was by the periodate-schiff technique of Di Ferrante et al (1971). Extracts were analysed for hydroxyproline by the Stegeman & Stalder technique (1967). * refers to analyses below the limits of detection. HA, CS and DS refer to the elution positions of standard hyaluronic acid, chondroitin sulphate and dermatan sulphate respectively, on cellulose acetate electrophoresis.

•approximately 17 gm dry weight isolated from approximately 400 bovine incisors.

The 2 M NaCl extract contained very little uronic acid positive material and from the strong absorbance at 260 nm (2.2 times that at 280 nm at pH 6.8) and from the loss of 80% of the carbazole positive material, after digestion with ribonuclease and deoxyribonuclease and subsequent dialysis, it was concluded to contain predominantly nucleic acid. However this extraction step was valuable in removing nucleic acids which otherwise would have contaminated the 4 M guanidinium chloride extract.

4.3 PURIFICATION AND CHARACTERIZATION

4.3.1 DEAE-cellulose Chromatography

A typical elution profile of the concentrated tissue extracts on DEAE-cellulose is shown in Fig. 17 and the analyses of the fractions obtained are given in Table 15. 85 - 98% of the uronic acid positive material applied to the DEAE-cellulose columns were recovered in the eluted fractions for all extracts studied except the 2 M NaCl extract of the ligament. Nearly all the collagen was eluted with 7 M urea and most of the uronic acid positive material was eluted with 2 M NaCl in 7 M urea (with the exception of the 2 M NaCl extract of the ligament). The presence of varying amounts of uronic acid positive material in the fractions eluted with 7 M urea and 0.15 M NaCl in 7 M urea is believed largely due to interference from non-proteoglycan material, in particular the nucleic acids. Cellulose acetate electrophoresis of these fractions of the 0.4 M guanidinium chloride extract of the skin and 0.1 M NaCl extract of the periodontal ligament revealed the presence of some material migrating with the mobility of standard hyaluronate. However most of the low salt fractions from the DEAE-cellulose column revealed no alcian blue positive material even though very high concentrations, based on the uronic acid analyses, were applied. These fractions were not analysed further. The anomalous behaviour of the 2 M NaCl extract is believed due to the presence of large amounts of nucleic acids and negligible quantities of proteoglycan.

The fractions eluted with 2 M NaCl in 7 M urea were dialysed extensively against distilled water then lyophilized. A slight turbidity was noticed prior to lyophilization of the 2 M NaCl eluted fraction of

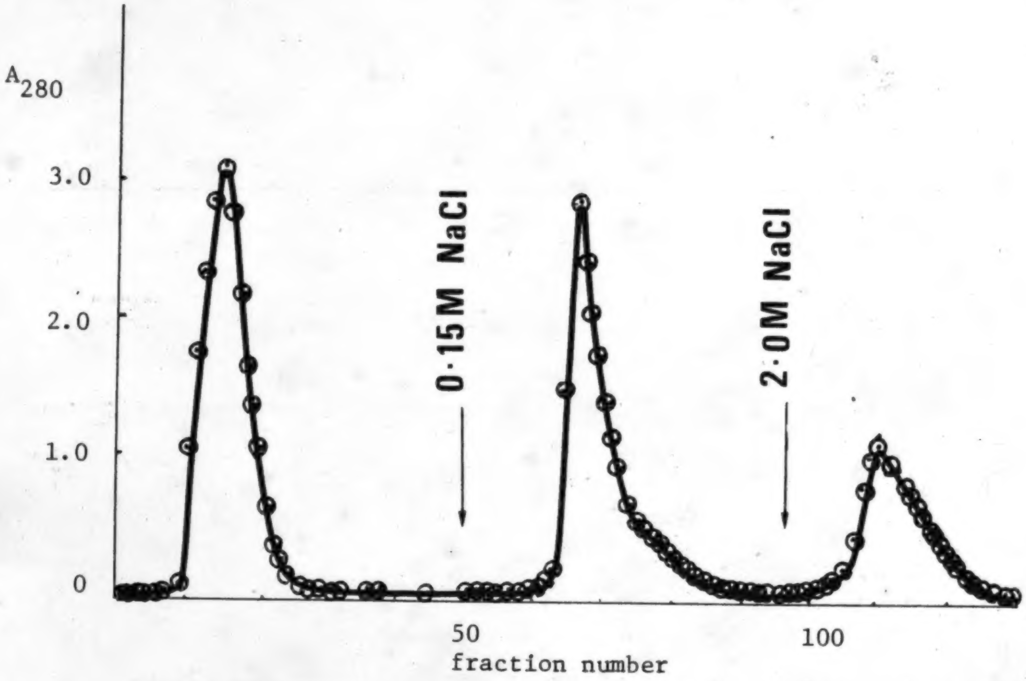


Fig. 17 Chromatography of the 4M Guanidinium Chloride Extract on DEAE-Cellulose

The 4M guanidinium chloride extract of the periodontal ligament was concentrated, made to 7M urea and applied to a DEAE-cellulose column, previously equilibrated with 7M urea, 0.05M Tris, pH 6.8. The column was eluted with 7M urea, 0.15M NaCl in 7M urea and 2.0M NaCl in 7M urea. Eluting solutions were buffered with 0.05M Tris pH 6.8. 2 ml fractions were collected and the absorbance at 280 nm measured.

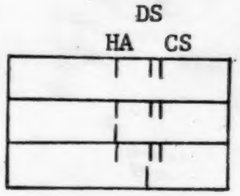
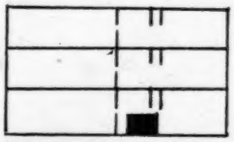
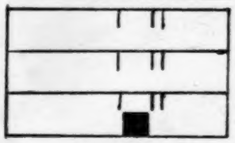
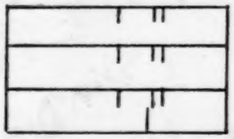
Fraction	Uronic Acid mg	Uronic Acid % total	Hyp mg	Cellulose Acetate Electrophoresis
Skin				
0.4 M GuCl 0.0	0.215	4.7		
0.4 M GuCl 0.15	0.275	6.1		
0.4 M GuCl 2.0	3.93	86.9		
4.0 M GuCl 0.0	0.246	6.1		
4.0 M GuCl 0.15	0.246	6.1		
4.0 M GuCl 2.0	3.23	80.8		
Periodontal Ligament				
0.10 NaCl 0.0	8.77	39.7	7.67	
0.10 NaCl 0.15	3.77	17.1	0.635	
0.10 NaCl 2.0	6.70	30.3	*	
2.0 NaCl 0.0	0.34	8.80	0.115	
2.0 NaCl 0.15	1.09	28.2	*	
2.0 NaCl 2.0	0.63	16.3	*	
4 M GuCl 0.0	1.48	13.9	7.32	
4 M GuCl 0.15	1.84	17.3	0.823	
4 M GuCl 2.0	6.36	60.0	*	

Table 15. Recovery of Uronic Acid and Hydroxyproline after DEAE-cellulose Chromatography of the Tissue Extracts

The 0.4 M and 4.0 M guanidinium chloride extracts of bovine skin and 0.1 M and 2.0 M NaCl extracts and 4.0 M guanidinium chloride extracts of bovine incisor periodontal ligament were concentrated, made to 7 M urea, 0.05 M Tris, pH 6.8 and applied to a DEAE-cellulose column previously equilibrated with 7 M urea, 0.05 M Tris, pH 6.8. The column was eluted with 7 M urea (fraction 0.0), 0.15 M NaCl in 7 M urea (fraction 0.15) and 2.0 M NaCl in 7 M urea (fraction 2.0). The recovery of the applied uronic acid and hydroxyproline were determined after dialysis against distilled water and papain digestion. HA, DS and CS refer to the elution positions of standard hyaluronic acid, dermatan sulphate and chondroitin sulphate respectively, on cellulose acetate electrophoresis. * refers to analyses below the limits of detection.

the 4 M guanidinium chloride extract of the periodontal ligament. This was removed by centrifugation and shown to contain 194 μg of hydroxyproline and 184 μg of uronic acid. The glycosaminoglycan, isolated by papain digestion was subjected to cellulose acetate electrophoresis and was shown to have a very similar mobility to that remaining in solution, though it appeared slightly more diffuse.

4.3.2 Density Gradient Centrifugation of Skin and Ligament Proteoglycans

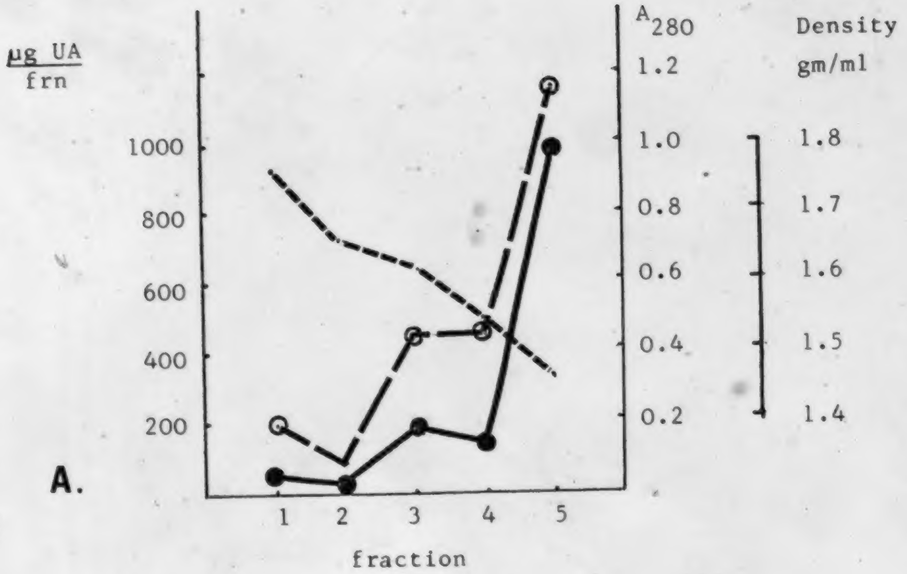
The proteoglycan extracted from bovine skin with 4 M guanidinium chloride and purified by DEAE-cellulose chromatography was subjected to density gradient centrifugation in 0.4 M guanidinium chloride at a starting density of 1.55 gm/ml. As observed with an apparently similar proteoglycan extracted from heart valves (Toole and Lowther, 1968b) more than 80% was found at the top of the gradient and appeared to have a buoyant density less than or equal to 1.46 gm/ml (Fig. 18a). Recentrifugation of this material at an initial density of 1.4 gm/ml and a guanidinium chloride concentration of 0.4 M gave the profile shown in Fig. 18b. The proteoglycan fractionated as a single component with a buoyant density between 1.4 and 1.5 gm/ml.

In light of these findings, the 4 M guanidinium chloride extract of the periodontal ligament, previously purified by DEAE-cellulose chromatography, was subjected to density gradient centrifugation in 4 M guanidinium chloride at a starting density of 1.4 gm/ml (Fig. 19b). A DEAE-cellulose purified skin proteoglycan preparation (4 M guanidinium chloride extract) was subjected to density gradient centrifugation under the same conditions in parallel (Fig. 19a). Both preparations ran as essentially single proteoglycan species. The analysis of dermatan sulphate closely paralleled that of the uronic acid, however the dermatan sulphate to uronic acid ratio varied slightly and was the greatest in the fraction containing the majority of the dermatan sulphate in both cases (Table 16). Some protein was separated from the proteoglycan and ran at the top of the gradient. Before centrifugation the skin and periodontal ligament preparations contained 71 and 61% protein respectively. After gradient centrifugation this was reduced to 64 and 51% respectively based on Lowry protein and uronic acid analyses.

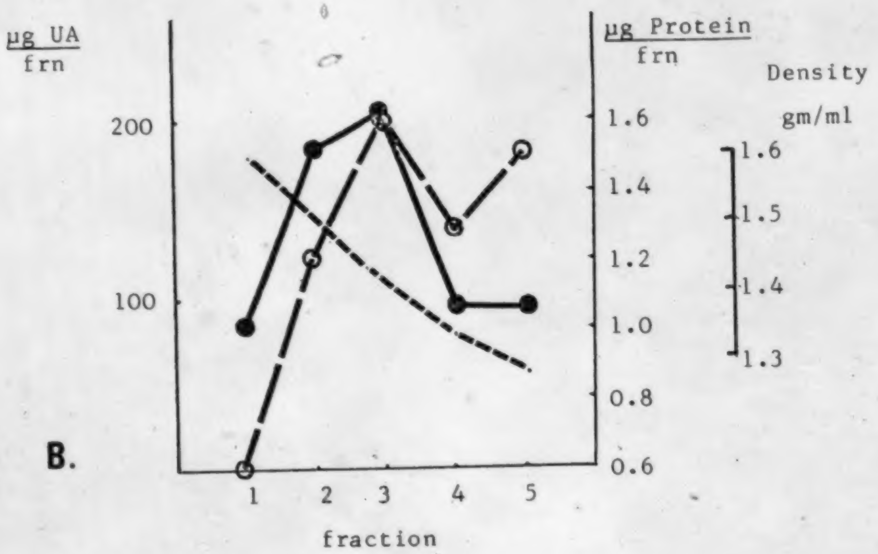
Fig. 18 Density Gradient Centrifugation of Skin Proteoglycan

(a) The 4 M guanidinium chloride extract of bovine skin was purified by DEAE-cellulose chromatography then made to a density of 1.55 gm/ml with CsCl, 0.4 M in guanidinium chloride and 0.05 M in sodium acetate, pH 5.8 and centrifuged at 190,000 g for 48 hrs. The gradient was divided into 5 equal fractions which were analysed for protein (○) by absorbance at 280 nm or by the Lowry technique after dialysis against distilled water, and for uronic acid (●) after dialysis and papain digestion. The density of the fractions was determined using a 100 µl constriction pipette as a pycnometer.

(b) Fractions 4 and 5 were made to a density of 1.44 gm/ml with CsCl, 0.4 M guanidinium chloride and 0.05 M sodium acetate, pH 5.8 and recentrifuged at 190,000 g for 48 hrs. This gradient was also divided into 5 equal fractions which were analysed for density, absorbance at 280 nm (○) and uronic acid content (●).


µg UA / frn
1000
800
600
400
200
A280
1.2
1.0
0.8
0.6
0.4
0.2
Density gm/ml
1.8
1.7
1.6
1.5
1.4
1
2
3
4
5
fraction

A.


µg UA / frn
200
100
µg Protein / frn
1.6
1.4
1.2
1.0
0.8
0.6
Density gm/ml
1.6
1.5
1.4
1.3
1
2
3
4
5
fraction

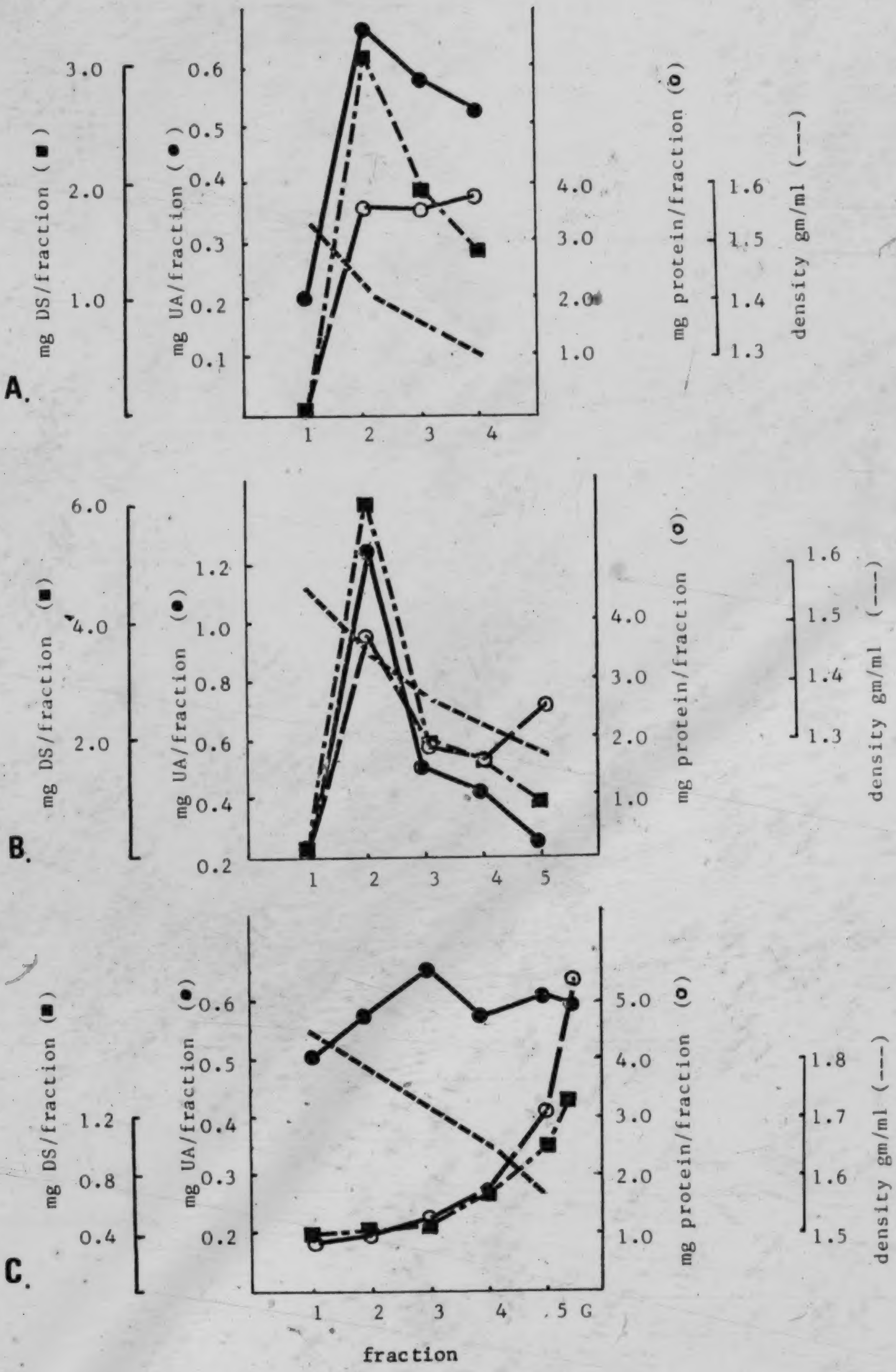
B.

Fig. 19 Density Gradient Centrifugation of Skin and Ligament Proteoglycans

(a) The proteoglycan extracted from bovine skin with 4 M guanidinium chloride purified by DEAE-cellulose chromatography was made to a density of 1.39 gm/ml with CsCl, 4 M in guanidinium chloride and 0.05 in sodium acetate, pH 5.8 and centrifuged at 190,000 g for 48 hrs. The gradient was divided into 4 approximately equal fractions (since fraction 5 in Table 16 was small it was included in fraction 4) which were analysed for density using a 100 µl constriction pipette as a pycnometer, protein (Lowry *et al*, 1951), dermatan sulphate (Di Ferrante *et al*, 1971) and uronic acid (Bitter and Muir, 1962).

(b) The proteoglycan extracted from the periodontal ligament with 4 M guanidinium chloride and purified by DEAE-cellulose chromatography was subjected to similar density gradient centrifugation except that the starting density was 1.41 gm/ml and the gradient was divided into 5 equal fractions.

(c) The proteoglycan extracted from bovine periodontal ligament and purified by DEAE-cellulose chromatography was subjected to a similar density gradient centrifugation except that the starting density was 1.63 gm/ml and guanidinium chloride concentration was 0.5 M. Five equal fractions plus a gel (G) which floated at the top of the gradient were obtained and analysed as described above.


A.
mg DS/fraction (■)
3.0
2.0
1.0
mg UA/fraction (●)
0.6
0.5
0.4
0.3
0.2
0.1
mg protein/fraction (o)
4.0
3.0
2.0
1.0
density gm/ml (---)
1.6
1.5
1.4
1.3
1
2
3
4
B.
mg DS/fraction (■)
6.0
4.0
2.0
mg UA/fraction (●)
1.2
1.0
0.8
0.6
0.4
0.2
mg protein/fraction (o)
4.0
3.0
2.0
1.0
density gm/ml (---)
1.6
1.5
1.4
1.3
1
2
3
4
5
C.
mg DS/fraction (■)
1.2
0.8
0.4
mg UA/fraction (●)
0.6
0.5
0.4
0.3
0.2
mg protein/fraction (o)
5.0
4.0
3.0
2.0
1.0
density gm/ml (---)
1.8
1.7
1.6
1.5
1
2
3
4
5
G
fraction

The skin proteoglycan preparation had a buoyant density of 1.42 gm/ml and the ligament proteoglycan, which gave a sharper fractionation, had a slightly higher buoyant density of 1.45 gm/ml. Alteration of the guanidinium chloride concentration from 0.4 to 4.0 M made little apparent difference to the density gradient centrifugation pattern of the skin proteoglycan (c.f. Fig. 18b and Fig. 19a) or to the protein content of the fractions. Fraction 3 (Fig. 18b) from the density gradient formed in 0.4 M guanidinium chloride had a protein content of 67% while fraction 2 (Fig. 19a) from the gradient formed in 4 M guanidinium chloride had a protein content of 64% based on Lowry protein and uronic acid analyses.

Densitometric scans from cellulose acetate electrophoresis of the glycosaminoglycans isolated from the various fractions of both the skin and ligament proteoglycans (4 M guanidinium chloride extracts), shown in Fig. 20, revealed only one sharp peak with a mobility slightly less than that of the standard dermatan sulphate and indiscernible from the pattern given by the 18 and 25% alcohol fractions isolated from the periodontal ligament by papain digestion. Since all fractions had essentially the same behaviour on cellulose acetate electrophoresis, a representative profile of the skin and ligament proteoglycan fractions only, is shown.

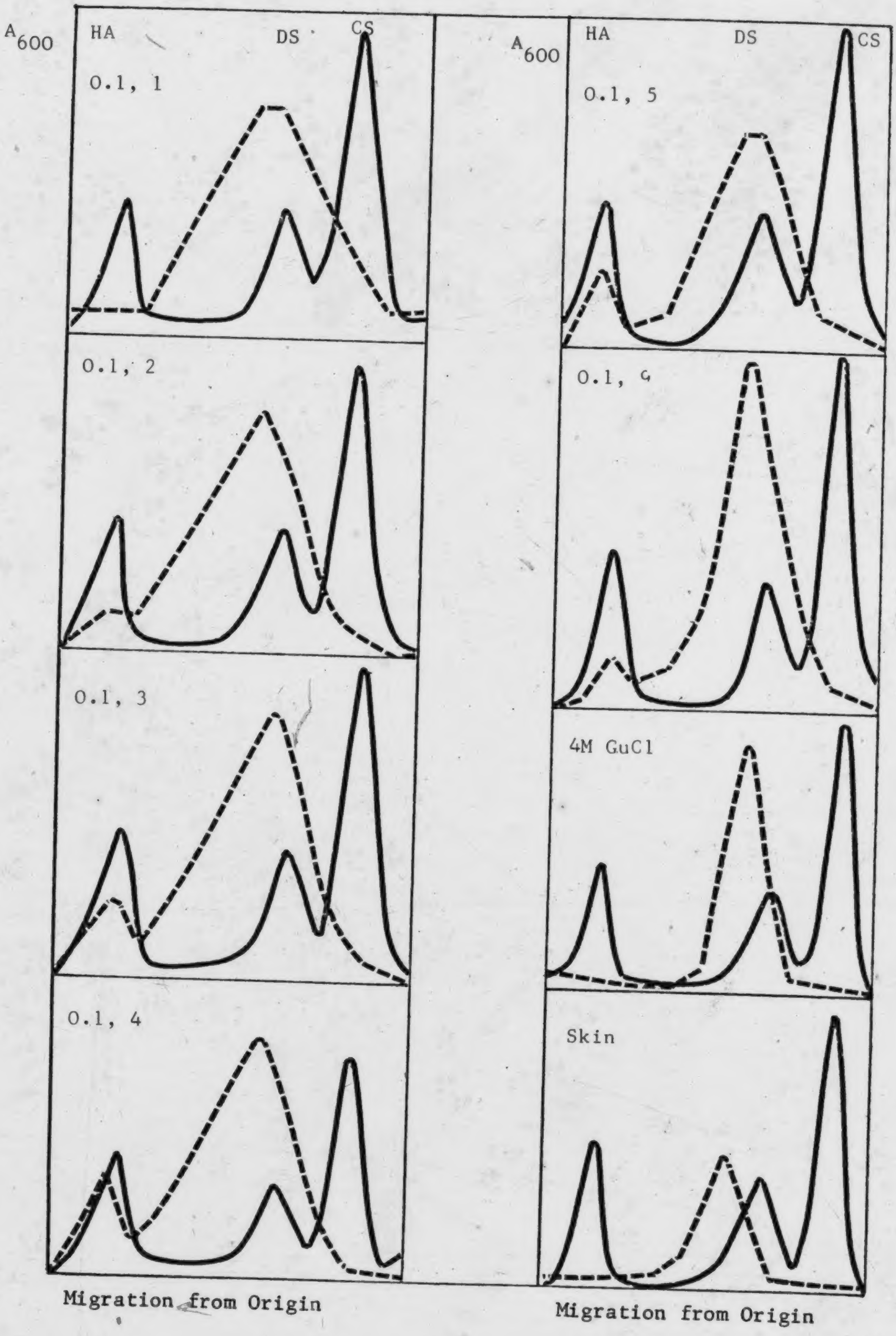
The 0.1 M NaCl extract of the periodontal ligament, after purification on DEAE-cellulose, was subjected to density gradient centrifugation in 0.4 M guanidinium chloride at an initial density of 1.63 gm/ml. The profile obtained, shown in Fig. 19c, suggests that most of this material has a buoyant density greater than 1.6 gm/ml, but the proteoglycan did not fractionate into a single species of a well defined buoyant density. The high density fractions contained low levels of protein and dermatan sulphate which were concentrated in the least dense fraction and the gel forming at the top of the gradient. These fractions contained 75% of the protein and 62% of the dermatan sulphate present (Table 16). Hydroxyproline analysis of the gel showed that collagen constituted only 4.5% of the total protein present in the gel (30 μg hydroxyproline equivalent to 0.22 mg collagen in the gel).

Densitometric scans from cellulose acetate electrophoresis of the

Fig. 20 Electrophoretic Patterns of Glycosaminoglycans from the Density Gradient Fractions of Skin and Ligament Proteoglycans

Glycosaminoglycans were isolated from the density gradient fractions of the skin and ligament proteoglycans (Fig. 19) by papain digestion, dialysed against distilled water then dried under vacuum. Glycosaminoglycan samples in distilled water (approx. 1.0 µg in uronic acid/µl) were subjected to cellulose acetate electrophoresis as described in section 2.2.5. The standard and sample glycosaminoglycan bands were scanned separately at 600 nm and the profiles superimposed.

0.1, 1; 0.1, 2; 0.1, 3; 0.1, 4; 0.1, 5 and 0.1, G refer to the glycosaminoglycans isolated from the density gradient fractions 1, 2, 3, 4, 5 and the gel of the 0.1 M NaCl extracted ligament proteoglycan. Representative profiles of glycosaminoglycans from the density gradient fractions of the 4 M guanidinium chloride extracted ligament proteoglycan (4 M GuCl) (gradient fraction 2) and 4 M guanidinium chloride extracted skin proteoglycan (skin) (gradient fraction 3) are also shown. HA, DS and CS refer to the position of standard hyaluronic acid, dermatan sulphate and chondroitin sulphate respectively. The solid lines are the profiles of the standard glycosaminoglycan mixtures and the dotted lines are the profiles of glycosaminoglycans from the density gradient fractions.


A600
HA
DS
CS
0.1, 1
0.1, 2
0.1, 3
0.1, 4
Migration from Origin
A600
HA
DS
CS
0.1, 5
0.1, 6
4M GuCl
Skin
Migration from Origin

Fraction		density mg/ml	protein mg/frn	uronic acid µg/frn	dermatan sulphate mg/frn	mg DS / mg UA	% protein
Skin	1	1.534	0.089	198	0.156	0.79	13.0
	2	1.422	3.57	663	3.12	4.70	64.2
	3	1.361	3.54	577	1.99	3.40	67.2
	4	1.304	3.74	525	1.39	2.65	70.4
	5	1.289	0.927	97.7	0.371	3.80	76.0
Ligament 4 M GuCl	1	1.567	0.432	258	0.583	2.26	35.8
	2	1.451	3.88	1255	6.10	4.85	50.8
	3	1.376	1.78	493	1.83	3.72	54.6
	4	1.327	1.68	422	1.64	3.88	57.0
	5	1.280	2.50	246	0.938	3.81	77.2
Ligament 0.1 M NaCl	1	1.791	0.342	449	0.158	0.35	20.2
	2	1.737	0.449	530	0.226	0.43	22.0
	3	1.670	0.767	605	0.310	0.51	29.7
	4	1.604	1.04	521	0.446	0.86	40.0
	5	1.514	2.53	559	0.783	1.40	60.1
	G		4.84	550	1.114	2.03	74.6

Table 16. Analyses of the Fractions from the Density Gradient Centrifugation of Skin and Ligament Proteoglycans

The 4 M guanidinium chloride extracts of bovine skin and periodontal ligament and the 0.1 M NaCl extract of periodontal ligament, previously purified by DEAE-cellulose chromatography were subjected to CsCl density gradient centrifugation at initial densities of 1.39, 1.41 and 1.63 gm/ml respectively, in guanidinium chloride (4 M for guanidinium chloride extracts and 0.4 M for the 0.1 M NaCl extract). The gradients were divided into 5 approximately equal fractions and analysed for density, using a 100 µl constriction pipette as a pycnometer, protein by the Lowry technique after dialysis, uronic acid (UA) by the carbazole technique, after dialysis and papain digestion, and dermatan sulphate (DS) by the Di Ferrante technique (1971), after dialysis. % protein was calculated from Lowry protein and uronic acid analyses, using a conversion factor of uronic acid to glycosaminoglycan of 3.3.

glycosaminoglycans, isolated from the various fractions, are shown in Fig. 20. All fractions contained a predominance of material which ran as a broad band, extending from the position of standard chondroitin sulphate to that of standard hyaluronic acid. In concurrence with the higher concentrations of dermatan sulphate found in the gel, glycosaminoglycans isolated from this fraction showed a sharper peak with a mobility slightly less than that of standard dermatan sulphate and similar to the 18 and 25% alcohol fractions isolated from the ligament by papain digestion. All fractions with the exception of the highest density fraction contained material which had a mobility very similar to that of standard hyaluronic acid.

4.3.3 Composite Agarose-Polyacrylamide Gel Electrophoresis of Density Gradient Fractions

The fractions isolated from density gradient centrifugation of the proteoglycans were subjected to composite agarose-polyacrylamide gel electrophoresis. Densitometric scans of gels stained for proteoglycan or protein are shown in Figs. 21, 22 and 23. The mobility of the bands observed relative to that of the marker dye (bromphenol blue) are shown in Table 17.

From the toluidine blue staining pattern, it appears that most of the gradient fractions of the skin proteoglycan contain two closely similar proteoglycan species with a relative mobility of 0.70 and 0.73. The proteoglycan bands were not always separated on gel electrophoresis. The reason for this variation is unclear but may be due to slight variations in electrophoresis conditions. Staining for protein showed three predominant bands, one with essentially identical mobility to one of the proteoglycan bands (0.70). Though it is difficult to make comparisons between bands distinguished with different dyes, these fractions appear to contain non-proteoglycan proteins of very similar mobility to the proteoglycans. The least dense fraction (5) shows the presence of much slower moving protein species, some of which failed to enter the gel. The most dense fraction (1) showed two bands of toluidine blue staining material which ran faster than the marker dye and failed to stain with Coomassie blue. This behaviour is consistent

Fig. 21 Densitometric Scans of Agarose-Polyacrylamide Gel Electrophoresis of Skin Proteoglycan Density Gradient Fractions

Samples (containing approximately 20 μg in uronic acid) from the density gradient fractions of skin proteoglycan were lyophilized then dissolved in 50 μl distilled water. 25 μl samples were subjected to electrophoresis on composite agarose-polyacrylamide gels (0.6% agarose, 2% acrylamide). Gels were stained for proteoglycan with toluidine blue (TB) and scanned at 550 nm, or for protein with Coomassie blue (CB) and scanned at 560 nm. Scans 1, 2, 3, 4 and 5 refer to the fractions isolated from the density gradient (Fig. 19). R_{bpb}, mobility relative to bromphenol blue.

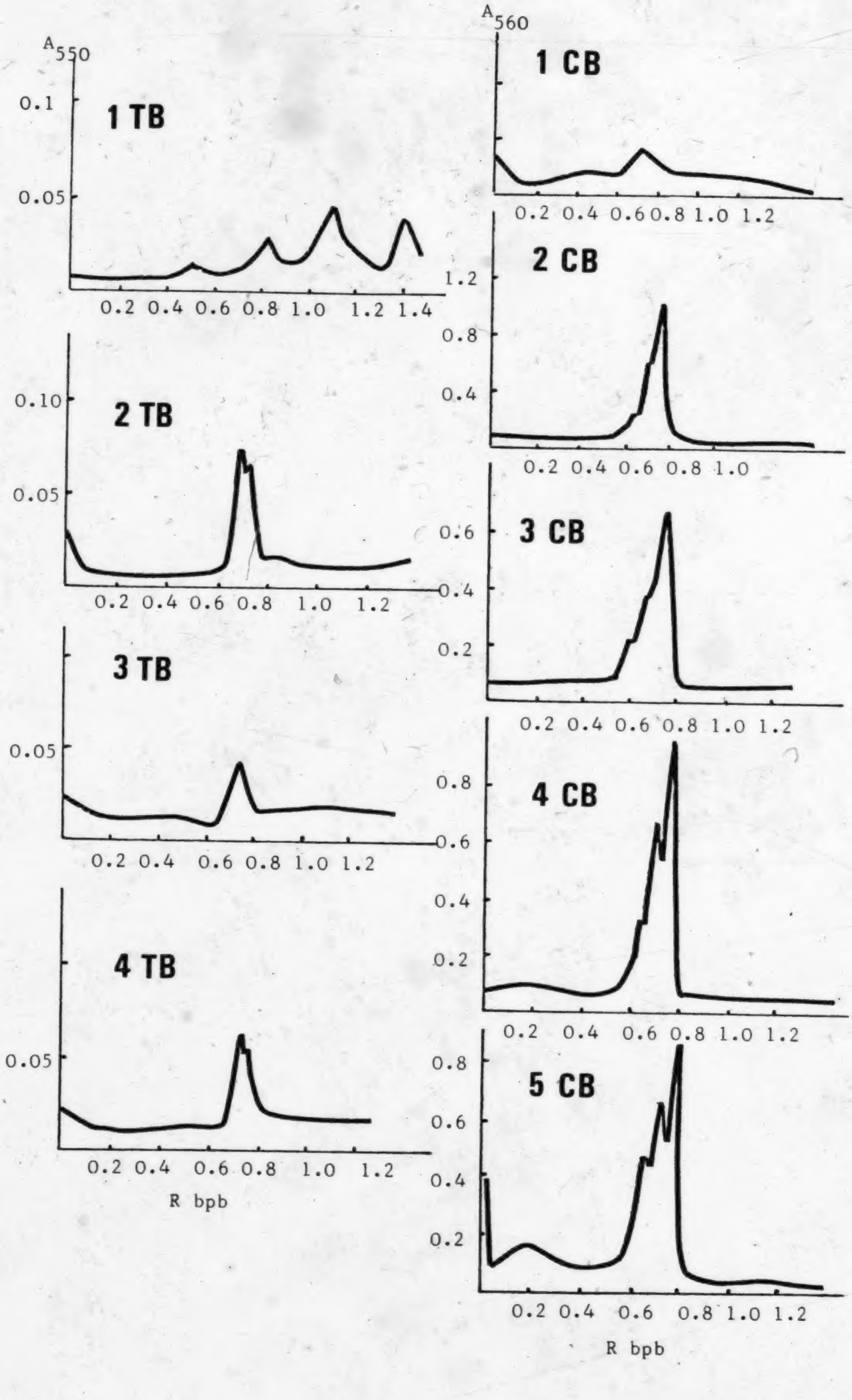

A550
0.1
0.05
1 TB
0.2 0.4 0.6 0.8 1.0 1.2 1.4
0.10
0.05
2 TB
0.2 0.4 0.6 0.8 1.0 1.2
3 TB
0.05
0.2 0.4 0.6 0.8 1.0 1.2
4 TB
0.05
0.2 0.4 0.6 0.8 1.0 1.2
R bpb
A560
1 CB
0.2 0.4 0.6 0.8 1.0 1.2
2 CB
1.2
0.8
0.4
0.2 0.4 0.6 0.8 1.0
3 CB
0.6
0.4
0.2
0.2 0.4 0.6 0.8 1.0 1.2
4 CB
0.8
0.6
0.4
0.2
0.2 0.4 0.6 0.8 1.0 1.2
5 CB
0.8
0.6
0.4
0.2
0.2 0.4 0.6 0.8 1.0 1.2
R bpb

Fig. 22 Densitometric Scans of Agarose-Polyacrylamide Gel Electrophoresis of 4 M Guanidinium Chloride Extracted Ligament Proteoglycan Density Gradient Fractions

Samples (containing approximately 20 µg uronic acid) from the density gradient centrifugation of 4 M guanidinium chloride extracted ligament proteoglycan were lyophilized then dissolved in 50 µl of distilled water. 25 µl samples were subjected to gel electrophoresis as described in Fig. 21. Scans 1, 2, 3, 4 and 5 refer to those fractions isolated from the density gradient. TB, toluidine blue stained. CB, Coomassie blue stained. R_{bpb}, mobility relative to bromphenol blue.

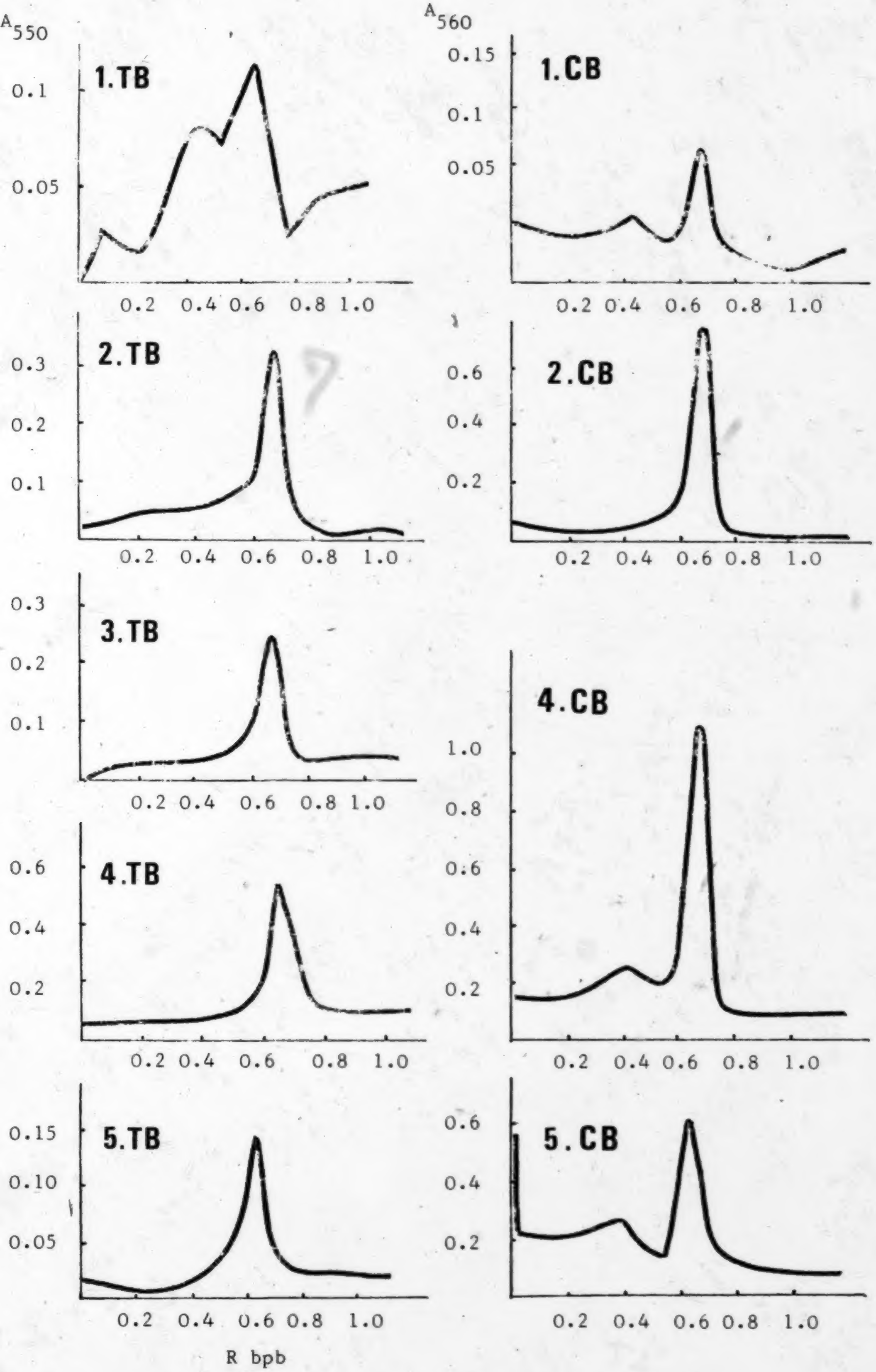

A550
1.TB
0.1
0.05
0.2 0.4 0.6 0.8 1.0
A560
1.CB
0.15
0.1
0.05
0.2 0.4 0.6 0.8 1.0
2.TB
0.3
0.2
0.1
0.2 0.4 0.6 0.8 1.0
2.CB
0.6
0.4
0.2
0.2 0.4 0.6 0.8 1.0
3.TB
0.3
0.2
0.1
0.2 0.4 0.6 0.8 1.0
4.CB
1.0
0.8
0.6
0.4
0.2
0.2 0.4 0.6 0.8 1.0
4.TB
0.6
0.4
0.2
0.2 0.4 0.6 0.8 1.0
5.TB
0.15
0.10
0.05
0.2 0.4 0.6 0.8 1.0
5.CB
0.6
0.4
0.2
0.2 0.4 0.6 0.8 1.0
R bpb

Fig. 23 Densitometric Scans of Agarose-Polyacrylamide Gel Electrophoresis of 0.1 M NaCl Extracted Ligament Proteoglycan

Samples (containing approximately 20 µg uronic acid) from the density gradient centrifugation of 0.1 M NaCl extracted ligament proteoglycans were lyophilized then dissolved in 50 µl of distilled water. 25 µl samples were subjected to gel electrophoresis as described in Fig. 21. Scans 1, 2, 3, 4, 5 and G (Gel) refer to those fractions isolated from the density gradient. PGS refers to a sample of proteoglycan subunit isolated from bovine nasal septum cartilage. TB, toluidine blue stained. CB, Coomassie blue stained. R_{bpb}, mobility relative to bromphenol blue.

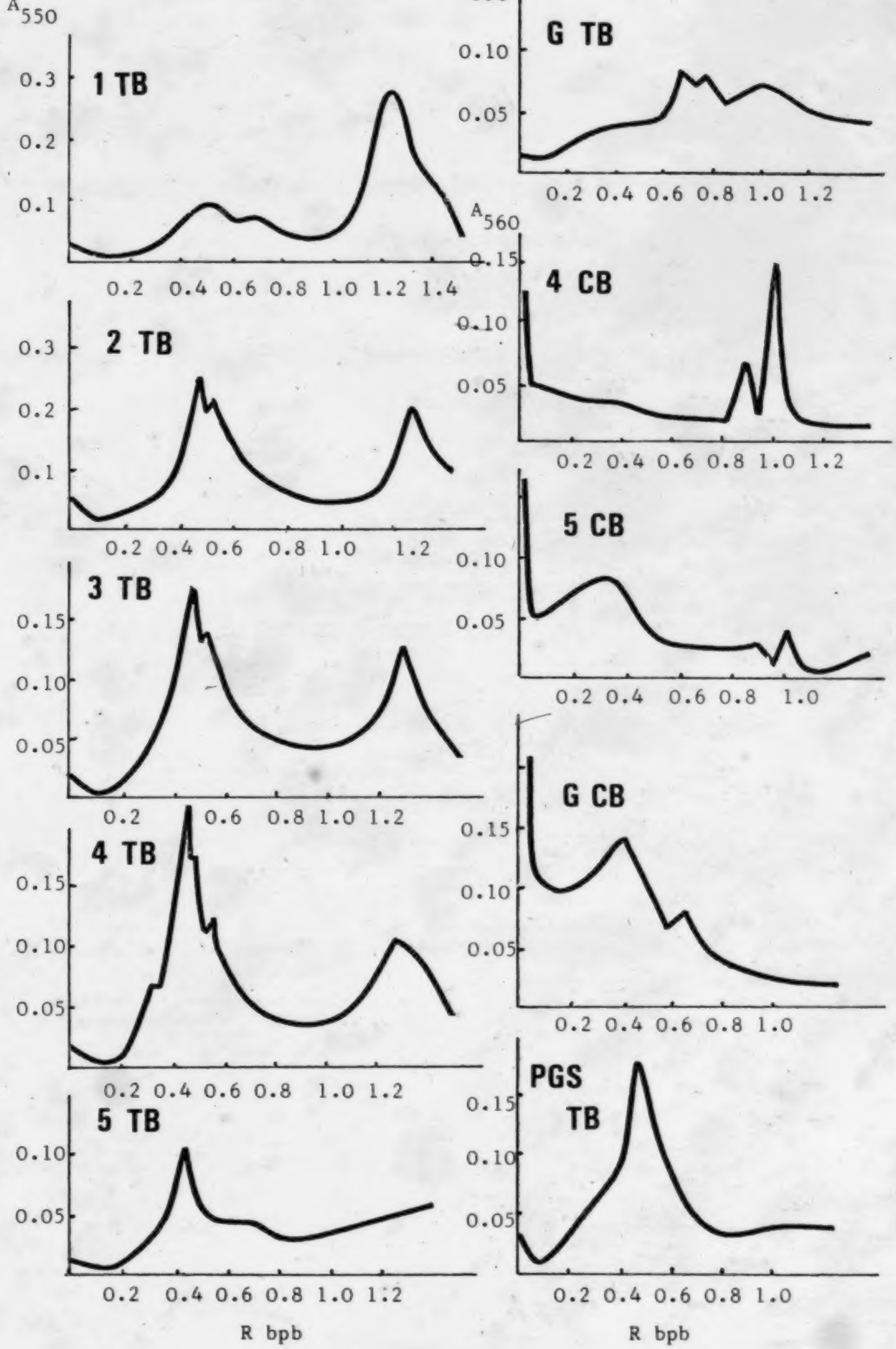

A_{550}
1 TB
0.3
0.2
0.1
0.2 0.4 0.6 0.8 1.0 1.2 1.4
2 TB
0.3
0.2
0.1
0.2 0.4 0.6 0.8 1.0 1.2
3 TB
0.15
0.10
0.05
0.2 0.6 0.8 1.0 1.2
4 TB
0.15
0.10
0.05
0.2 0.4 0.6 0.8 1.0 1.2
5 TB
0.10
0.05
0.2 0.4 0.6 0.8 1.0 1.2
R bpb
A_{550}
G TB
0.10
0.05
0.2 0.4 0.6 0.8 1.0 1.2
A_{560}
4 CB
0.15
0.10
0.05
0.2 0.4 0.6 0.8 1.0 1.2
5 CB
0.10
0.05
0.2 0.4 0.6 0.8 1.0
G CB
0.15
0.10
0.05
0.2 0.4 0.6 0.8 1.0
PGS TB
0.15
0.10
0.05
0.2 0.4 0.6 0.8 1.0
R bpb

Table 17. Gel Electrophoretic Properties of Skin and Ligament Density Gradient Fractions

Proteoglycans extracted from bovine skin and periodontal ligament and purified by DEAE-cellulose chromatography were subjected to cesium chloride density gradient centrifugation. The electrophoretic behaviour of the various fractions, on composite polyacrylamide-agarose gel electrophoresis, is shown in Figs. 21, 22 & 23. Gels were stained for protein (Coomassie blue) and for proteoglycan (toluidine blue) and the mobility of the bands (observed visually) relative to that of the marker dye (bromphenol blue) is given. Skin proteoglycan was that extracted with 4 M guanidinium chloride (GuCl). Fractions 1 to 5 are those obtained from density gradient centrifugation of the proteoglycans and shown in Fig. 19. * refers to very faint bands.

Fraction	Relative Mobility	
	Toluidine Blue	Coomassie Blue
Skin Proteoglycan		
1	0.73*, 0.83*, 1.10, 1.44	0.71
2	0.70, 0.73, 0.84*	0.64, 0.72, 0.76
3	0.70, 0.73*	0.62, 0.70, 0.75
4	0.71, 0.73	0.63, 0.70, 0.75
5	0.70, 0.73	0.0, 0.17, 0.64 0.70, 0.76
Periodontal Ligament 4 M GuCl Proteoglycan		
1	0.45, 0.64	0.43, 0.65
2	0.66	0.66
3	0.66	
4	0.66	0.39, 0.66
5	0.64	0.0, 0.39, 0.64 0.79*
Periodontal Ligament 0.1 M NaCl Proteoglycan		
1	0.47, 0.70, 1.23	
2	0.44, 0.51, 1.24	
3	0.45, 0.52, 1.25	
4	0.32, 0.45, 0.47, 0.55, 1.26	0.0, 0.37*, 0.43* 0.87, 0.99
5	0.30*, 0.43, 0.47* 0.70	0.0, 0.43, 0.87, 0.99
Gel	0.38, 0.66, 0.78	0.0, 0.39, 0.65
Cartilage Proteoglycan		
subunit	0.30, 0.45	
aggregate	0.0, 0.40	

Table 17. Gel Electrophoretic Properties of Skin and Ligament Density Gradient Fractions

with the presence of free glycosaminoglycan chains or nucleic acids.

The gradient fractions of the 4 M guanidinium chloride extracted ligament proteoglycan all showed the predominance of a single species of proteoglycan which had a relative mobility of 0.64 to 0.66 and stained with both Coomassie blue and toluidine blue (Fig. 22, Table 17). The most dense fraction (1) also contained a proteoglycan species which had a relative mobility of 0.43 to 0.45 and the less dense fractions showed the presence of lower mobility protein species, some of which failed to enter the gel.

The gel electrophoretic profiles of the gradient fractions of the 0.1 M NaCl extracted ligament proteoglycans were much more complex. Gel electrophoresis displayed the presence of a mixture of slow moving proteoglycan components as well as some fast moving components (Fig. 23). The predominant proteoglycan species had a mobility similar to the major component found on the electrophoresis of proteoglycan subunit isolated from bovine nasal septum cartilage (Fig. 23 and Table 17). Coomassie blue did not stain these components of the higher density fractions (1, 2 and 3). The protein present was probably masked by glycosaminoglycan chains and unavailable to Coomassie blue. A similar staining pattern was observed with cartilage proteoglycans. A number of protein species were observed in the lower density fractions and a large proportion of these failed to enter the gel. The gel floating at the top of the density gradient contained two proteoglycan species. One stained with toluidine blue and Coomassie blue and had a similar mobility to the proteoglycan extracted with 4 M guanidium chloride (relative mobility 0.65 - 0.66). The other was detected with toluidine blue only and had a relative mobility of 0.78. The other apparent peaks on the scan (Fig. 23 GTB) were not obvious visually and are believed due to uneven gel destaining.

4.3.4 Molecular Characteristics of the 4 M Guanidinium Chloride Extracted Proteoglycans

4.3.4.1 Gel Chromatography

The elution profile obtained when skin proteoglycan, purified by DEAE-cellulose chromatography and density gradient centrifugation, was chromatographed on Sepharose 2-B, is shown in Fig. 24a. A single peak

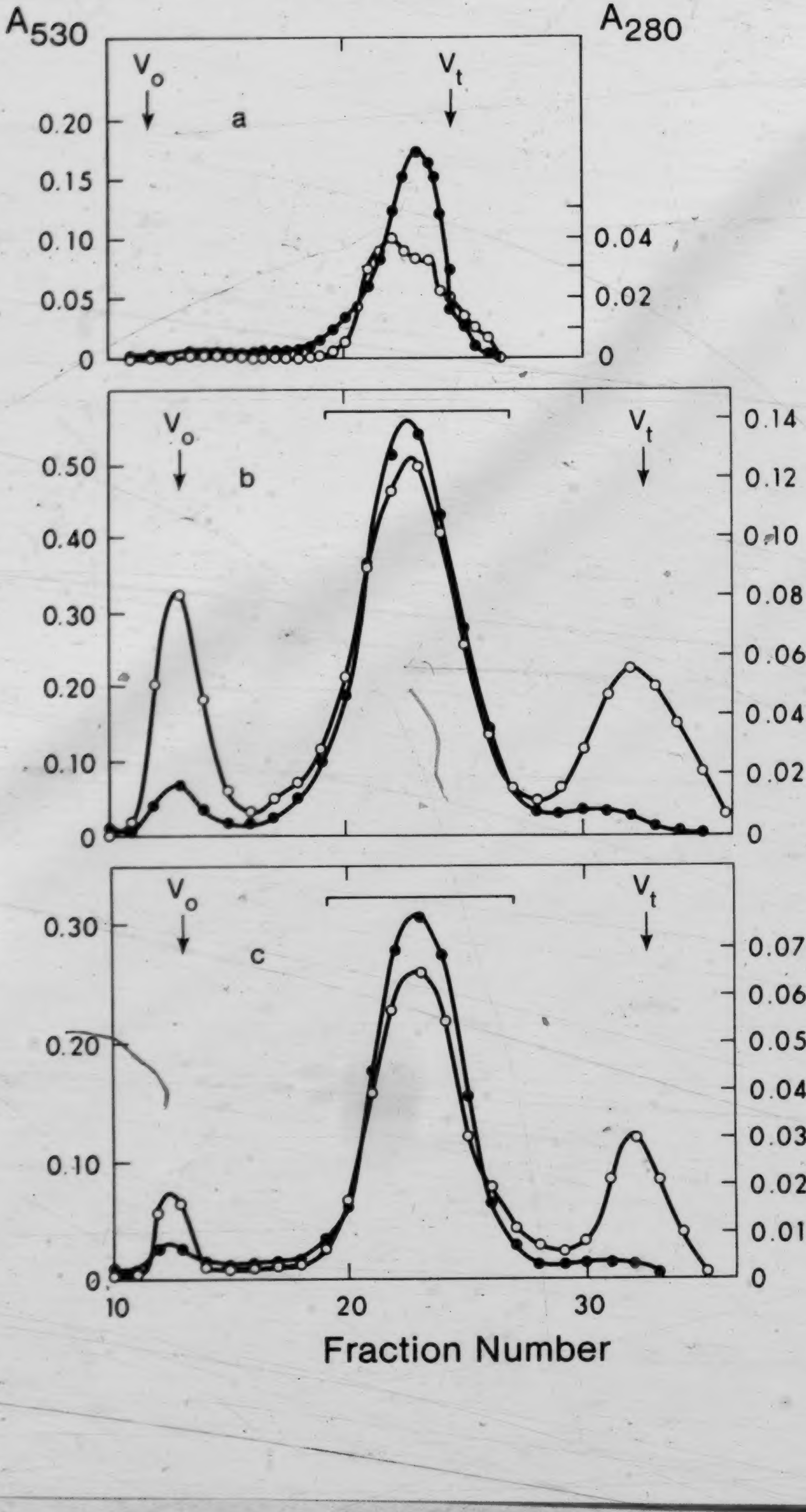
Fig. 24 Gel Chromatography of Skin Proteoglycan

(a) Proteoglycan (approximately 1 mg) extracted from bovine skin with 4 M guanidinium chloride and purified by DEAE-cellulose chromatography and density gradient centrifugation pooled fractions 2, 3 and 4, (Fig. 18b) was applied to a Sepharose 2-B column (1 x 100 cm), equilibrated with 0.5 M sodium acetate, pH 6.8 and eluted with the same buffer at 4 ml/hr.

(b) Proteoglycan (2.5 mg) extracted from bovine skin and purified by DEAE-cellulose chromatography was applied to a Sepharose 6-B column (1 x 90 cm) and eluted with the same buffer at 4 ml/hr.

(c) Density gradient fractions 2 and 3 (Fig. 19a) of the skin proteoglycan were pooled and an aliquot (1.6 mg) subjected to gel chromatography on the Sepharose 6-B column under the same conditions.

2 ml fractions were collected and measured for absorbance at 280 nm (○) and for uronic acid by the manual (a) or automated (b) and (c) carbazole technique (●). Vo is the elution position of cartilage proteoglycan aggregate and Vt the elution position of ^3H_2O. The bar in (c) refers to the fractions taken for isolation and analyses of glycosaminoglycans. The peaks marked with bars in both (b) and (c) were pooled and analysed for protein content by the Lowry technique (1951). Aliquots of the peak marked in (c) were used for amino acid analysis and aliquots of the peak marked in (b) were used for the determination of the molecular weight by sedimentation equilibrium centrifugation.


A530
A280
a
b
c
Vo
Vt
0.20
0.15
0.10
0.05
0
0.04
0.02
0.50
0.40
0.30
0.14
0.12
0.10
0.08
0.06
0.02
0.07
0.05
0.03
0.01
10
20
30
Fraction Number

very close to the total volume of the column, was observed by monitoring the distribution of protein and uronic acid. Since no higher molecular weight material, such as that isolated from pig skin (Öbrink, 1972), was observed in these preparations, subsequent chromatography of the 4 M guanidinium chloride extracted proteoglycans from bovine skin and periodontal ligament was performed on Sepharose 6-B.

Density gradient centrifugation made little difference to the elution profiles of the skin proteoglycan on Sepharose 6-B (Fig. 24b and c). The material taken prior to density gradient centrifugation shows the presence of contaminating protein near the void and total volumes of the column and well separated from the proteoglycan peak. After density gradient centrifugation no change was observed in the elution profile of the proteoglycan, however there is a reduction in the contaminating protein peaks.

A somewhat similar situation was found with 4 M guanidinium chloride extracted ligament proteoglycan. Gel chromatography prior to density gradient centrifugation showed the presence of a prominent proteoglycan peak and protein peaks at the void volume and near the total volume of the column, however in this case a shoulder of uronic acid positive material was observed that eluted close to the void volume of the column (Fig. 25a). After density gradient centrifugation there was little change in the major proteoglycan peak, but the protein peaks at the void and near the total volumes of the column were markedly diminished as was the shoulder of uronic acid positive material near the void volume of the column.

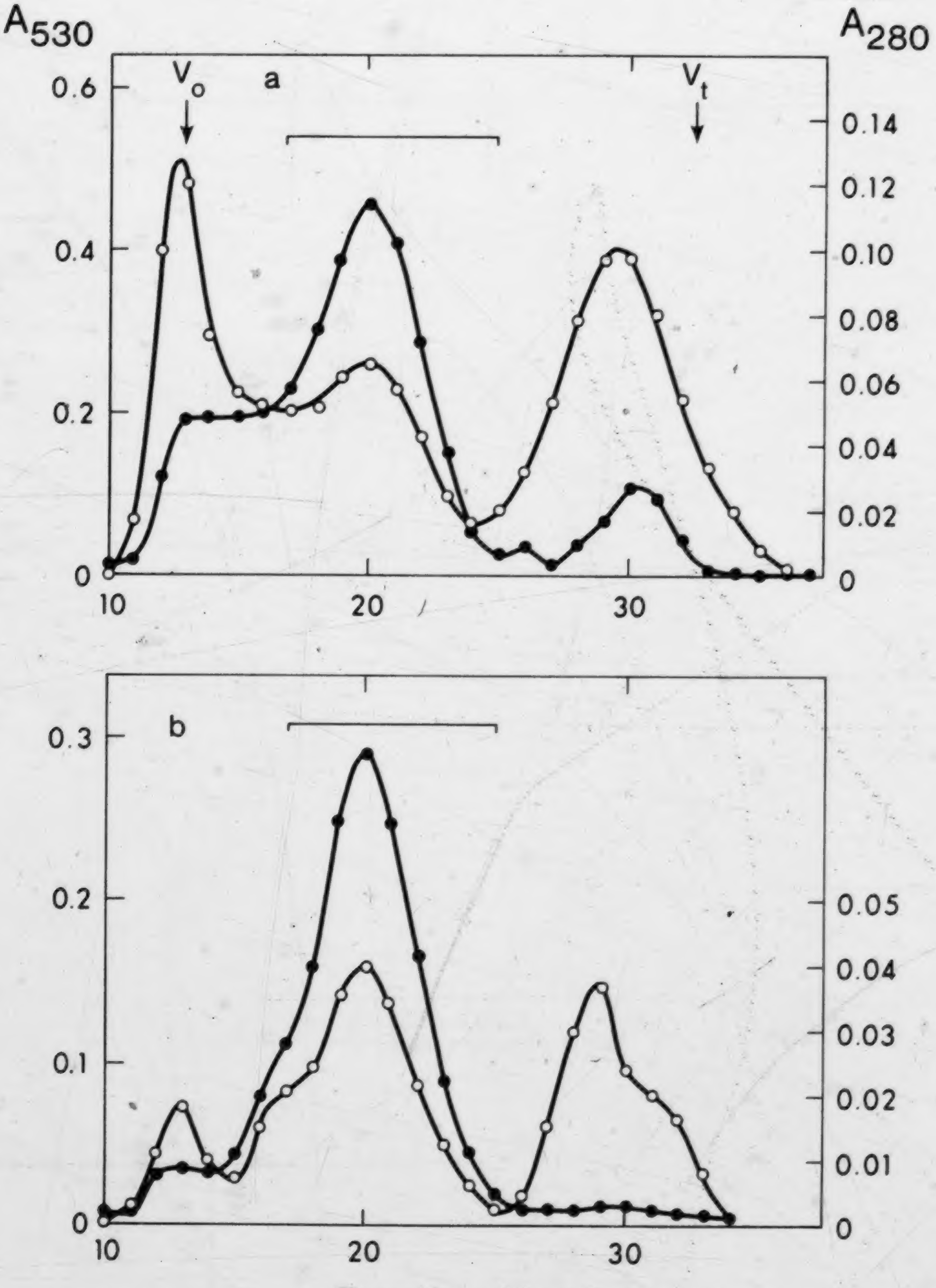
Equilibrium density gradient centrifugation in 4 M guanidinium chloride causes the dissociation of cartilage proteoglycan aggregate to free hyaluronic acid chains, link proteins and proteoglycan subunit (Hascall and Sajdera, 1969, Hardingham and Muir, 1974). However a similar treatment of the 4 M guanidinium chloride extracted ligament or skin proteoglycans, after purification by DEAE-cellulose chromatography caused neither change in apparent molecular size nor change in the protein content of the proteoglycans. The skin proteoglycan peak (Fig. 24b) was shown to contain 61.0% protein and 39.0% glycosaminoglycan by uronic acid and Lowry protein analysis. After density gradient centrifugation in 4 M guanidinium chloride the proteoglycan, further

Fig. 25 Gel Chromatography of 4 M Guanidinium Chloride Extracted Periodontal Ligament Proteoglycans

(a) Proteoglycan (approximately 2 mg) extracted from bovine periodontal ligament with 4 M guanidinium chloride and purified by DEAE-cellulose chromatography was applied to a Sepharose 6-B column (1 x 90 cm) and eluted with 0.5 M sodium acetate, pH 6.8 at 4 ml/hr.

(b) Density gradient fractions 2 and 3 of the same ligament proteoglycan were pooled and an aliquot (approximately 2 mg) was applied to the Sepharose 6-B column and eluted as described above.

2 ml fractions were collected in both cases and analysed for absorbance at 280 nm (○) and for uronic acid (●) by the automated carbazole technique. Vo is the elution position of aggregated cartilage proteoglycan and Vt the elution position of ^3H_2O. The bar in (b) refers to the fractions taken for the isolation and analyses of glycosaminoglycans. The peaks marked with bars in both (a) and (b) were pooled and analysed for protein content by the Lowry technique (1951). Aliquots of the peak marked in (b) were used for amino acid analyses and aliquots of the peak marked in (a) were used for the determination of molecular weight by sedimentation equilibrium centrifugation.


A530
A280
a
V_o
V_t
0.6
0.4
0.2
0
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0
10
20
30
b
0.3
0.2
0.1
0
0.05
0.04
0.03
0.02
0.01
0
10
20
30
Fraction Number

purified by Sepharose 6-B gel chromatography (Fig.24c), was shown to contain 60.8% protein and 39.2% glycosaminoglycan. Similar analysis of the 4 M guanidinium chloride extracted ligament proteoglycan showed that before gradient centrifugation the proteoglycan obtained from gel chromatography on Sepharose 6-B (Fig.25a) contained 47.3% protein and 52.7% glycosaminoglycan, and that obtained from gel chromatography after density gradient centrifugation (Fig.25b) contained 47.7% protein and 52.3% glycosaminoglycan. Thus either these proteoglycans do not appear to have a higher order of association such as is observed with cartilage proteoglycans or any associated protein or polysaccharide is lost on DEAE-cellulose chromatography.

4.3.4.2 Molecular Weight

The weight average molecular weights of the skin and ligament proteoglycans after purification by gel chromatography (peaks marked in Fig. 24b and 25a respectively) were determined by sedimentation equilibrium centrifugation to be 100,000 and 130,000 respectively. However, whereas sedimentation velocity studies showed the ligament proteoglycan to be homogeneous, the skin proteoglycan showed the presence of a small amount of lower molecular weight contaminant. An attempt was made to compensate for this by ignoring the first two points taken across the cell and using the last 8 points only to calculate molecular weight. The lower molecular weight contaminant was believed to have arisen due to the action of an endogenous protease (believed to be present in these preparations, see section 4.5) on the proteoglycan while the proteoglycan was incubated at 20° C for several hours due to a centrifuge malfunction.

4.3.4.3 Amino Acid Analysis

Analysis of the 4 M guanidinium chloride extracted skin and periodontal ligament proteoglycans (aliquots taken from gel chromatography shown in Figs. 24c and 25b) showed that they had virtually identical amino acid compositions (Table 18). The only residue that showed substantial difference was phenylalanine and, as mentioned in section 2.2.12.8, this amino acid could only be roughly estimated due to the poor separation from galactosamine. The amino acid analysis of these proteoglycans showed familial resemblance to the dermatan sulphate

Table 18. Amino Acid Composition of Dermatan Sulphate Proteoglycans

The periodontal ligament (P.L.) and skin proteoglycans were extracted with 4 M guanidinium chloride and purified by DEAE-cellulose chromatography, and gel chromatography (aliquots of peaks shown in Figs. 25b and 24c). The amino acid composition was determined as described in section 2.2.12.8. Each value given is the average of two separate analyses on duplicate hydrolysates. For comparison, analyses are given for pig skin proteoglycan extracted with hot 6 M urea and purified by density gradient centrifugation (Öbrink, 1972), heart valve proteoglycan purified by the same technique (Toole and Lowther, 1968b), and tendon proteoglycan extracted with 3 M $MgCl_2$ and purified by affinity chromatography and density gradient centrifugation (Anderson, 1975). Analysis of the keratan sulphate proteoglycan reported by Axelsson and Heinegard (1978) is also included for comparison.

Amino Acid	Residues per 1000 residues Dermatan Sulphate Proteoglycans					Keratan Sulphate Proteoglycan
	P.L.	Bovine Skin	Pig Skin	Bovine Heart Valve	Bovine Tendon	
Hyp	0	0	-	14	-	-
Asp	126	129	134	122	110	153
Thr	40	42	46	56	43	35
Ser	76	71	69	67	79	74
Glu	105	106	110	139	104	106
Pro	67	72	79	75	87	62
Gly	84	85	81	90	89	48
Ala	51	52	53	77	53	43
Val	59	62	56	50	58	52
Met	8	8	14	5	10	11
Ileu	57	61	56	37	54	47
Leu	126	126	132	96	119	158
Tyr	29	28	24	23	22	40
Phe	31	17	34	33	28	34
Hyl	0	0	-	-	-	-
Lys	76	83	29	46	86	63
His	28	28	22	19	29	21
Arg	32	29	38	51	29	27

Table 18. Amino Acid Composition of Dermatan Sulphate Proteoglycans

proteoglycans isolated from pig skin, bovine heart valves and bovine tendon (Table 18), though some marked differences were observed. Analysis of the pig skin and bovine heart valve preparations showed relatively low levels of lysine, probably arising from the carbamylation of the ε-amino group during isolation. It has been shown (Hagel et al, 1971 and Gerding et al, 1971) that cyanate formation in urea solutions cannot be avoided at pH values higher than 4, and is greatly favoured by increasing temperature. The skin and heart valve preparations cited were extracted with 6 M urea at 60° C and were thus exposed to considerable amounts of cyanate, which is known to carbamylate free amino groups, resulting in the formation of homocitrulline (Stark et al, 1960). The bovine skin and periodontal ligament proteoglycans were also exposed to concentrated urea solutions, however the temperature was always maintained at or below 4° C and the solutions were buffered with 0.05 M Tris which should have reacted with any cyanate formed. Lysine levels similar to those of the dermatan sulphate proteoglycan isolated without exposure to urea (bovine tendon preparation, Anderson, 1975) suggest that modification of the lysine residues in these preparations was negligible. The other differences in amino acid composition observed may have been due to different techniques of isolation and purification of the proteoglycans.

Comparison of the amino acid analyses of the dermatan sulphate proteoglycans with that of a keratan sulphate proteoglycan (Axelsson and Heinegård, 1978) showed some resemblance, suggesting that the protein cores of these distinctly different proteoglycans may have a familial similarity.

4.3.5 Molecular Characteristics of the 0.1 M NaCl Extracted Periodontal Ligament Proteoglycan

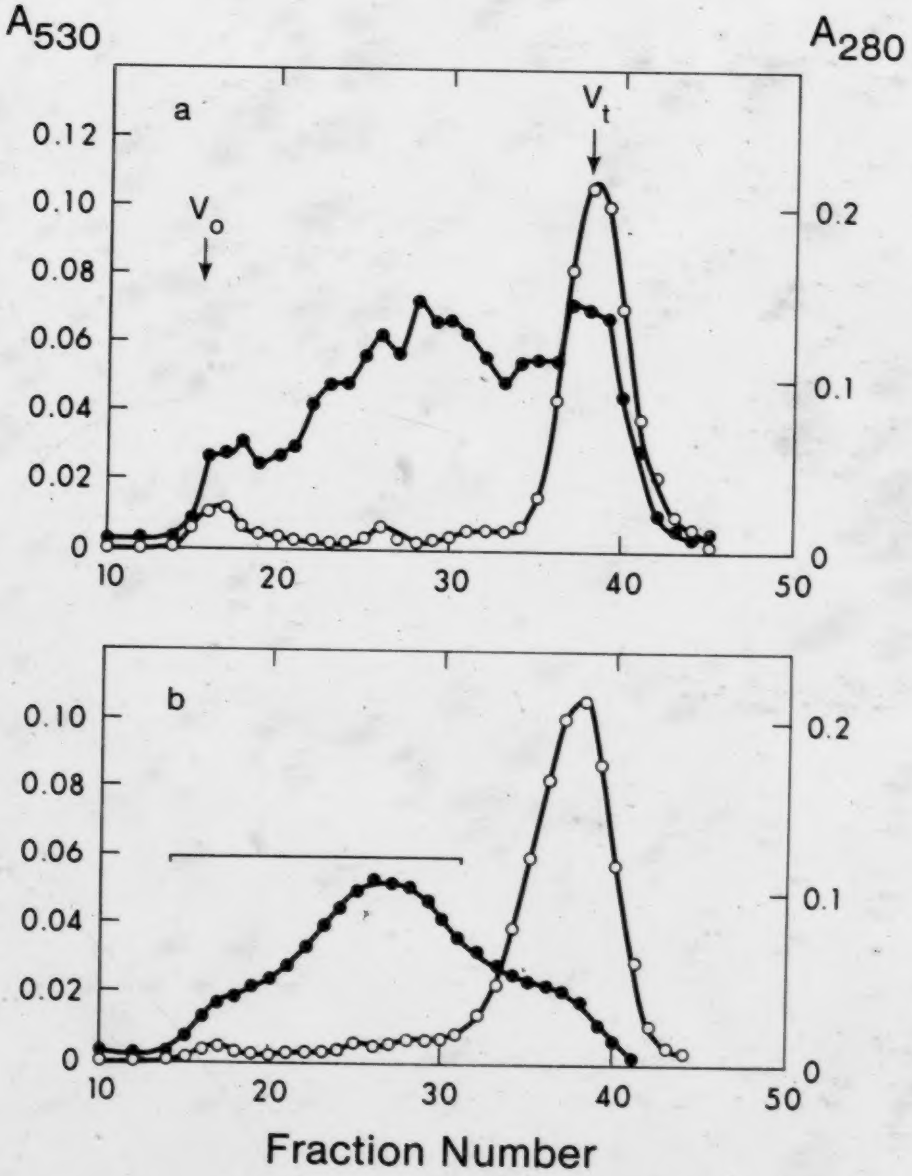
As was observed with gel electrophoresis, the gel chromatographic behaviour of the 0.1 M NaCl extracted ligament proteoglycan was much more complex and from the elution behaviour on Sepharose 2-B of the DEAE-cellulose purified preparation, shown in Fig.26a, it appears to contain a number of proteoglycan species of varying molecular size. Furthermore from the relative absorbance at 260 and 280 nm (2.3 at pH 6.8) the material eluting near the total volume of the column appears to be

Fig. 26 Gel Chromatography of 0.1 M NaCl Extracted Periodontal Ligament Proteoglycans

(a) Proteoglycan (approximately 1.5 mg) extracted from bovine periodontal ligament with 0.1 M NaCl and purified by DEAE-cellulose chromatography was applied to a Sepharose 2-B column (1 x 90 cm) and eluted with 0.5 M sodium acetate, pH 6.8 at 4 ml/hr.

(b) Density gradient fractions 2, 3 and 4 of the DEAE-cellulose purified, 0.1 M NaCl extracted ligament proteoglycan were pooled and an aliquot (approximately 1.0 mg) was applied to a Sepharose 2-B column and eluted as described above.

2 ml fractions were collected and analysed for absorbance at 280 nm (○) and for uronic acid by the automated carbazole technique (●). Vo refers to the elution position of aggregated cartilage proteoglycan and Vt the elution position of ^3H_2O. The bar in (b) refers to the fractions taken for the isolation and analysis of glycosaminoglycans and analysis of amino acid composition.


A530
A280
a
b
V_t
V_o
0.12
0.10
0.08
0.06
0.04
0.02
0
0.2
0.1
0
10
20
30
40
50
Fraction Number

heavily contaminated with nucleic acids.

Fractions 2, 3 and 4 from the equilibrium density gradient centrifugation of the 0.1 M NaCl extracted ligament proteoglycan were pooled and applied to the Sepharose 2-B column (Fig. 26b). The elution behaviour was similar to that obtained prior to density gradient centrifugation, though it appeared slightly less complex and enriched in higher molecular weight material. Contaminating nucleic acids were still present, though the lower molecular weight proteoglycans co-eluting with this material were considerably diminished and thus most likely present in the lower density fractions of the gradient.

Analysis showed that this higher molecular weight proteoglycan preparation (aliquot from gel chromatography, Fig.26b) had an amino acid composition resembling the cartilage proteoglycans (Table 19). This similarity is consistent with the behaviour of the proteoglycans on Sepharose 2-B gel chromatography. The amino acid composition of the other chondroitin sulphate type proteoglycans quoted (Table 19) showed marked differences particularly in the serine, aspartic acid and glycine residues. This probably arises from the apparently greater heterogeneity of the aorta (Antonopoulos et al, 1974) and cornea (Axelsson and Heinegård, 1975) preparations and the much smaller size of the heart valve preparation (Lowther et al, 1970). However all the noncartilagenous chondroitin sulphate-type proteoglycans showed much higher levels of lysine than the cartilage preparations.

It may be noted from Table 19, that the two proteoglycan preparations isolated from the periodontal ligament (that is, the 0.1 M NaCl extracted and 4 M guanidinium chloride extracted proteoglycans) showed distinct differences in amino acid composition and, though it remains to be proven, they appear to be different gene products.

4.3.6 Glycosaminoglycan Composition of the Proteoglycans

The glycosaminoglycans of the proteoglycans isolated from bovine skin, periodontal ligament and cartilage and from the residue obtained after guanidinium chloride extraction of the periodontal ligament are shown in Table 20.

The proteoglycans extracted from bovine skin and periodontal ligament with 4 M guanidinium chloride contained a predominance of glycosaminoglycans

Table 19. Amino Acid Composition of Chondroitin Sulphate Proteoglycans

The periodontal ligament proteoglycans (0.1 M NaCl extracted proteoglycan and 4 M guanidinium chloride extracted proteoglycan included for comparison) were aliquots taken from gel chromatography (shown in Fig. 25b and 26b). The amino acid composition was determined as described in section 2.2.12.8 . Analyses of cartilage proteoglycans NDI non-aggregating and DI aggregating were taken from Muir and Hardingham, 1975. The aorta proteoglycan analyses are taken from Antonopoulos et al, 1974. The proteoglycan preparation was a 4 M guanidinium chloride extract purified by DEAE-cellulose chromatography. The analyses of the heart valve chondroitin sulphate proteoglycan are taken from Lowther et al, 1970. The proteoglycan was extracted with 1.0 M NaCl and purified by density gradient centrifugation. The cornea proteoglycan analyses were reported by Axelsson and Heinegård (1975). The proteoglycan was extracted by 4 M guanidinium chloride, purified by DEAE-cellulose chromatography and separated from the keratan sulphate proteoglycan (Table 18) by alcohol precipitation. 0 refers to analyses below the level of quantitation.

Amino Acid	Residues per 1000 Residues						Periodontal Ligament 4 M GuCl
	Periodontal Ligament 0.1 M NaCl	Cartilage		Aorta	Heart Valve CS-PG	Cornea	
		NDI	DI				
Hyp	0	-	-	-	-	-	0
Asp	79	68	76	105	100	101	126
Thr	75	58	62	86	92	37	40
Ser	150	150	123	104	99	58	76
Glu	152	146	140	131	135	106	105
Pro	59	80	99	74	75	84	67
Gly	131	143	130	85	71	178	84
Ala	67	72	79	58	67	87	51
Val	51	62	59	58	59	58	59
Met	6	4	3	12	10	2	8
Ileu	31	39	37	44	37	39	57
Leu	54	81	77	96	74	84	126
Tyr	28	13	19	19	22	19	29
Phe	44	25	28	35	35	30	31
Hyl	0	-	-	-	-	-	0
Lys	33	10	12	35	53	53	76
His	17	8	10	27	21	18	28
Arg	23	29	44	34	33	48	32

Table 19. Amino Acid Composition of Chondroitin Sulphate Proteoglycans

precipitating at low alcohol concentrations characteristic of glycosaminoglycans rich in iduronic acid. The glycosaminoglycans of the skin proteoglycan tended to precipitate at slightly higher alcohol concentration consistent with their lower molecular weight (Fransson and Rodén, 1967b). The periodontal ligament proteoglycans extracted with 0.1 M NaCl, however, contained predominantly glycosaminoglycans precipitating at higher alcohol concentrations characteristic of the glycosaminoglycans rich in glucuronic acid. This proteoglycan also contained substantial amounts of glycosaminoglycans precipitated by CPC from 0.03 M NaCl and 0.15 M $MgCl_2$, indicative of the presence of hyaluronic acid and heparan sulphate or undersulphated chondroitin sulphate. As would be expected, the glycosaminoglycans isolated from cartilage proteoglycan precipitated in a manner characteristic of chondroitin 4-sulphate. The residue obtained after extraction of the periodontal ligament gave a profile of glycosaminoglycans which differed from that obtained with the extractable material and was suggestive of another distinct type of proteoglycan(s).

Thus the 4 M guanidinium chloride extracted skin proteoglycan appears to consist of a single species which has a buoyant density of approximately 1.42 gm/ml, a protein content of 61%, an elution coefficient (Kav) of 0.51 on Sepharose 6-B, a relative mobility of 0.71 - 0.74 on composite agarose polyacrylamide gel electrophoresis and a molecular weight of 100,000. The two bands of proteoglycan observed on gel electrophoresis did not always separate and are difficult to explain, though highly purified disaggregated cartilage proteoglycan preparations also give two bands on similar gel electrophoresis (Roughley, 1977 and Stanescu and Maroteaux, 1975). The glycosaminoglycans in the skin proteoglycan appear to be predominantly of the type rich in iduronic acid.

The proteoglycan extracted from the periodontal ligament with 4 M guanidinium chloride is similar to that extracted from the skin, however the glycosaminoglycan chains are larger (30×10^3 compared with 18×10^3). The larger molecular size of the glycosaminoglycan chains probably accounts for the higher buoyant density (1.45 gm/ml), a lower protein content (47%) and larger size (molecular weight 130,000, Kav on Sepharose 6-B of 0.36, relative mobility of 0.64 - 0.66 on composite gel

Table 20. Glycosaminoglycan Composition of Bovine Skin, Ligament and Cartilage Proteoglycans

Proteoglycan was extracted from bovine skin and periodontal ligament and purified by DEAE-cellulose chromatography, density gradient centrifugation and gel chromatography as described in section 2.2.6.2. The fractions from gel chromatography (shown in Figs. 24 and 25) and cartilage proteoglycan subunit (prepared as described in section 2.2.6.1) were subjected to alkali cleavage and reduction with NaB^3H_4 and the glycosaminoglycans recovered as described in the methods section 2.2.1. Glycosaminoglycans from the guanidinium chloride residue were recovered after papain digestion by CPC precipitation (0.03 and 0.15 M $MgCl_2$ fractions) and subsequent alcohol precipitation (18, 25, 40 and 50% alcohol fractions). The fractions obtained from the proteoglycans and residue were analysed for uronic acid (the results are expressed as a percentage of the total uronic acid recovered) and radioactivity. The number average molecular weights calculated (as shown in section 2.2.3) from these results are given. * refers to molecular weights calculated after gel chromatography of the glycosaminoglycans on Sephadex G-200. The other molecular weights were calculated from analysis of the alcohol precipitates. The CPC fractions were those precipitated from 0.3 M NaCl and 0.15 M $MgCl_2$ (see Fig. 5). GuCl, guanidinium chloride.

Proteoglycan	CPC Fraction	Alcohol Fraction	Uronic Acid %	$\bar{M}n \times 10^{-3}$
Skin	0.03		0	
4 M GuCl extract	0.15		0	
		18	18.4	
		25	49.2	18.2*
		40	29.8	17.0
		50	2.6	
Periodontal Ligament	0.03		0	
4 M GuCl extract	0.15		4.7	
		18	62.3	30.8*
		25	18.1	27.3
		40	12.7	29.8
		50	2.3	
Periodontal Ligament	0.03		23.1	
0.1 M NaCl extract	0.15		16.4	
		18	0	
		25	0	
		40	26.1	29.8*
		50	34.4	21.0*
Periodontal Ligament	0.03		2.8	
Residue	0.15		29.6	
		18	2.1	
		25	33.6	
		40	25.2	
		50	6.9	
Cartilage	0.03		0	
Subunit	0.15		9.0	
		18	0	
		25	0	
		40	81.7	21.0*
		50	9.3	

electrophoresis) of the ligament proteoglycans.

The periodontal ligament proteoglycan extracted with 0.1 M NaCl contains predominantly higher molecular weight material which has a higher buoyant density, lower protein content and a predominance of glycosaminoglycans rich in glucuronic acid.

There appears to be some overlap in the material extracted from the periodontal ligament with 0.1 M NaCl and 4 M guanidinium chloride. The 0.1 M NaCl extract contains some material similar in size and iduronic acid content to that extracted by 4 M guanidinium chloride, consistent with the work of Pearson et al (1975) who found that approximately 25% of the total dermatan sulphate in the periodontal ligament could be extracted with 0.15 M NaCl. Similarly the 4 M guanidinium chloride extract contains some higher molecular size material.

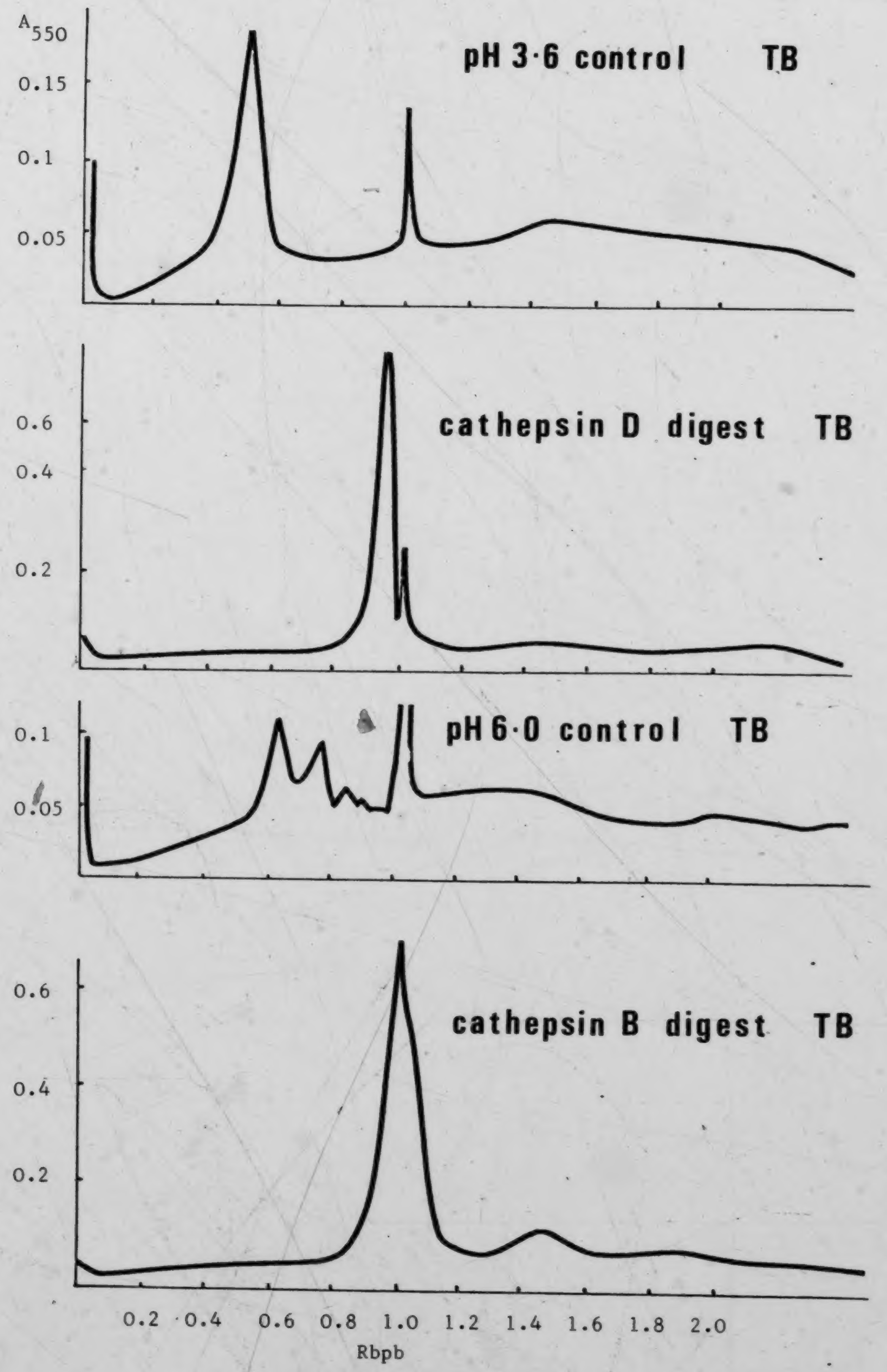
4.4 THE DEGRADATION OF SKIN AND LIGAMENT PROTEOGLYCANS BY TISSUE PROTEINASES

In order to further investigate the structure of the skin and periodontal ligament proteoglycans and their susceptibility to digestion by a number of tissue proteinases partially purified preparations (2 M NaCl fractions from DEAE-cellulose chromatography) were digested with cathepsins D and B and leucocytic elastase and the products electrophoresed on composite agarose polyacrylamide gels (Fig. 27, 28 and 29, and Table 21). The proteinases used in this investigation produced final degradation products having a range of sizes.

The fragments produced by cathepsin B and elastase digestion of the 4 M guanidinium chloride extracted skin and ligament proteoglycans had relative mobilities only slightly less than that of the free glycosaminoglycan chains, suggesting that they contain single glycosaminoglycan chains linked to small peptides (Fig. 27 and 28). In addition to these fragments, faint fast moving bands were also detected with toluidine blue. These bands were not present in the unincubated proteoglycans and were consistently found with this type of incubation. They may be due to the presence of small glycosaminoglycan or oligosaccharide chains attached either to the proteoglycans or, more likely, to contaminating glycoproteins, or proteoglycans since they were not observed after digestion of the highly purified proteoglycans.

Fig. 27 Composite Gel Electrophoresis of Tissue Protease Digests of Skin Proteoglycan

Bovine skin proteoglycan extracted with 4 M guanidinium chloride and purified by DEAE-cellulose chromatography was incubated with cathepsin D or B or leucocytic elastase at 37° C for 24 hrs. Control incubations were performed in the appropriate buffer in the absence of enzyme. The buffer used in cathepsin D digestions was 0.2 M PAF, pH 3.6. The enzyme concentrations was 0.3 μg per incubation or .006 μg/μg of PG. The buffer used in cathepsin B digestions was 0.1 M Na K phospate which contained 1 mM EDTA and 1 mM cysteine, pH 6.0 and an enzyme concentration of 6 μg per incubation or .07 μg/μg of PG was employed. The buffer used in leucocytic elastase incubations was 0.05 M Tris HCl containing 1 M KCl, pH 7.5 and the enzyme concentration was 6 μg per incubation or .07 μg/μg of PG. The products of digestion were subjected to composite agarose-polyacrylamide gel electrophoresis (0.6% agarose, 2% polyacrylamide) and stained for glycosaminoglycans with toluidine blue (TB) or for protein with Coomassie blue (CB). The gels were scanned at 550 nm (toluidine blue stained) or 560 nm (Coomassie blue stained). R_{bpb}, mobility relative to bromphenol blue.


A550
0.15
0.1
0.05
pH 3·6 control TB
0.6
0.4
0.2
cathepsin D digest TB
0.1
0.05
pH 6·0 control TB
0.6
0.4
0.2
cathepsin B digest TB
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
Rbpb

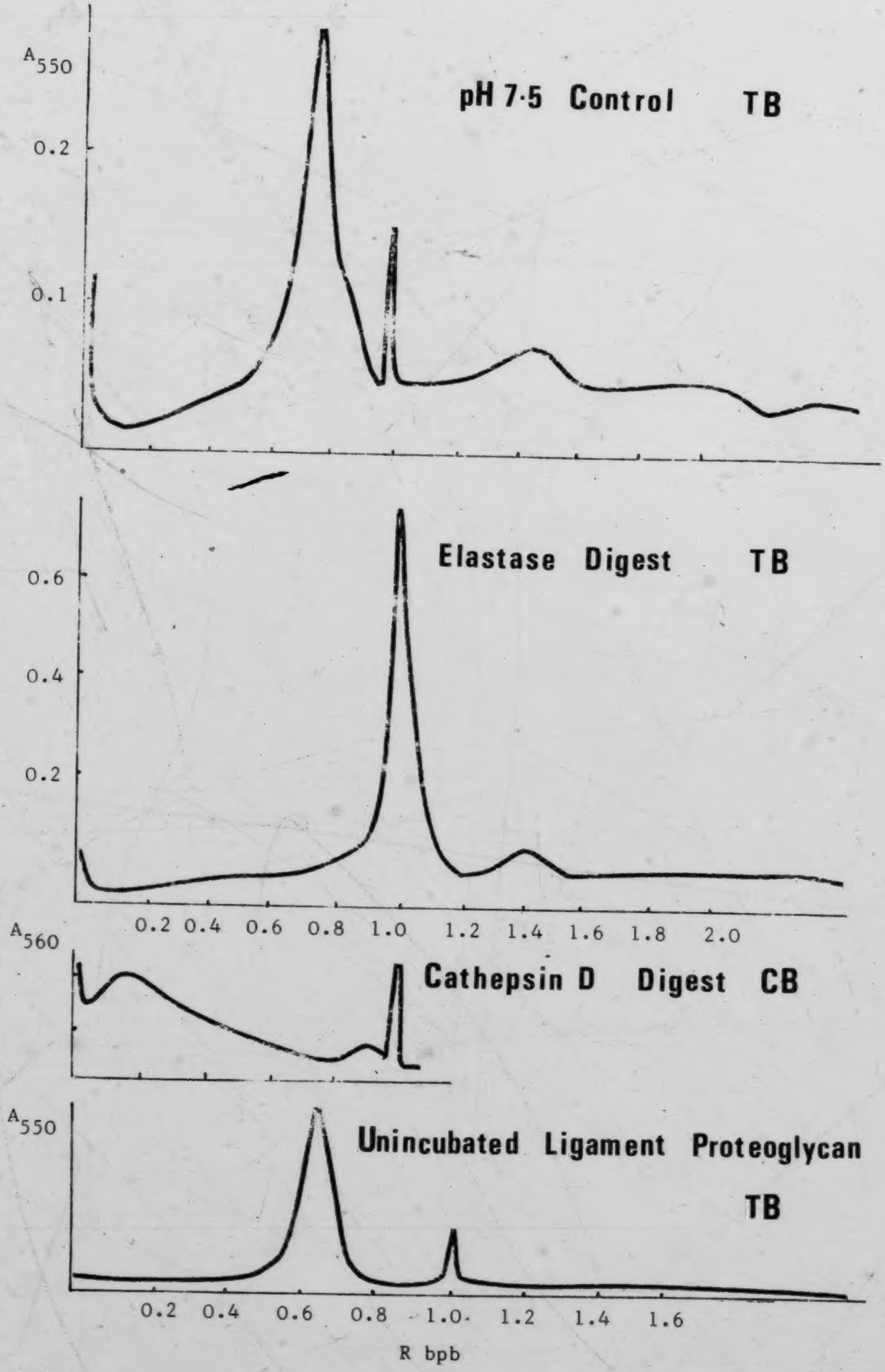
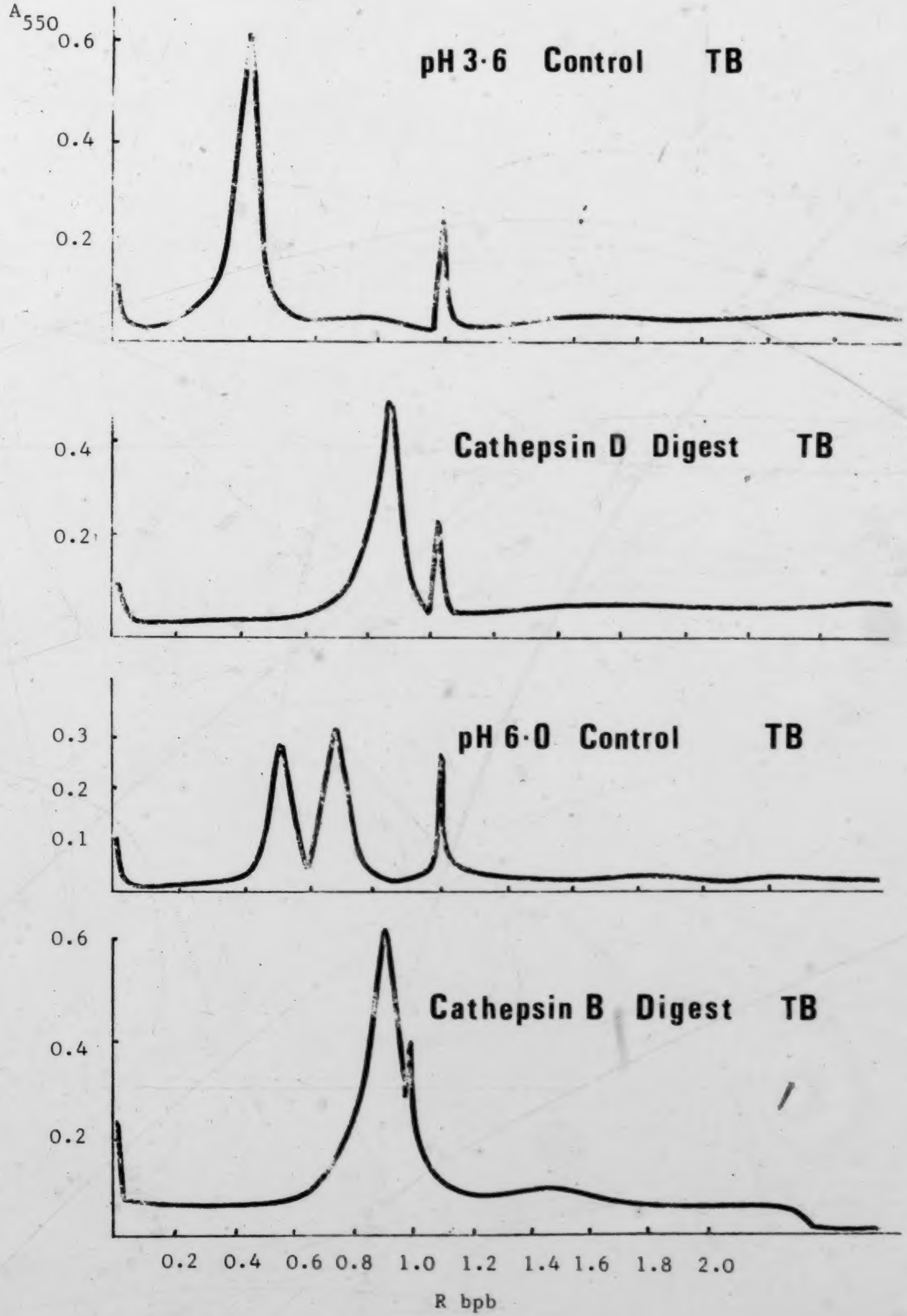

A_{550}
0.2
0.1
pH 7·5 Control TB
0.6
0.4
0.2
Elastase Digest TB
0.2 0.4 0.6 0.8 1.0 1.2 1.4 1.6 1.8 2.0
A_{560}
Cathepsin D Digest CB
A_{550}
Unincubated Ligament Proteoglycan
TB
0.2 0.4 0.6 0.8 1.0 1.2 1.4 1.6
R bpb

Fig. 28 Composite Gel Electrophoresis of Tissue Protease Digests of 4 M Guanidinium Chloride Extracted Ligament Proteoglycan

Proteoglycan isolated from the bovine periodontal ligament by extraction with 4 M guanidinium chloride and purified by DEAE-cellulose chromatography was subjected to protease digestion followed by composite agarose-polyacrylamide gel electrophoresis as described in the previous figure. R_{bpb}, mobility relative to bromphenol blue. TB, toluidine blue. CB, Coomassie blue.


A_{550}
0.6
0.4
0.2
pH 3·6 Control TB
0.4
0.2
Cathepsin D Digest TB
0.3
0.2
0.1
pH 6·0 Control TB
0.6
0.4
0.2
Cathepsin B Digest TB
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
R bpb

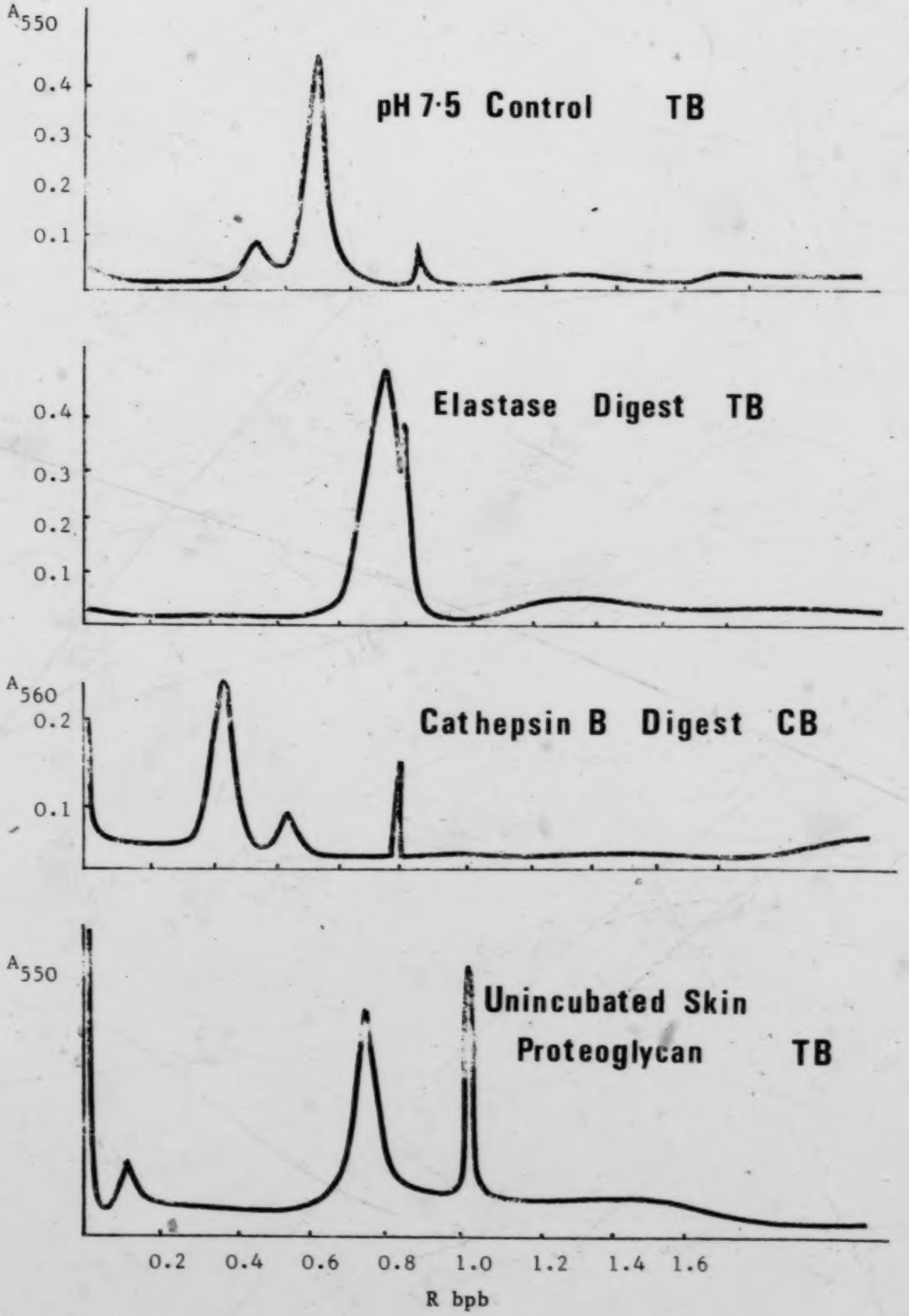
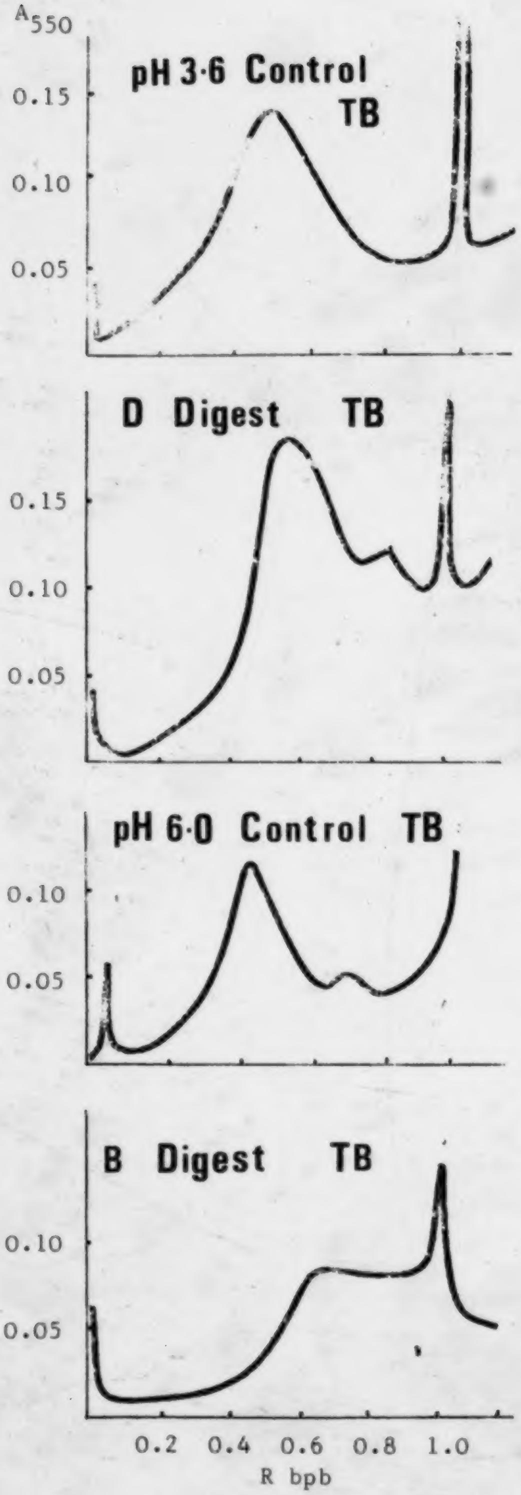
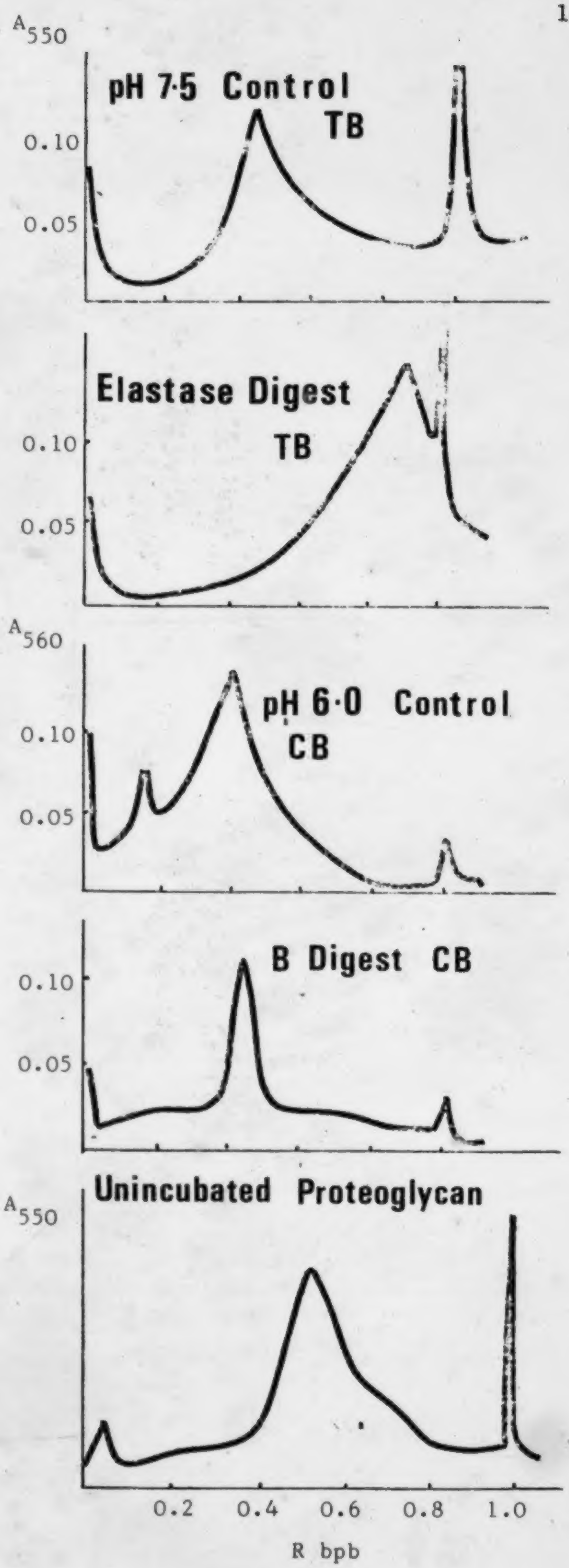

A_{550}
0.4
0.3
0.2
0.1
pH 7·5 Control TB
0.4
0.3
0.2
0.1
Elastase Digest TB
A_{560}
0.2
0.1
Cathepsin B Digest CB
A_{550}
Unincubated Skin Proteoglycan TB
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
R bpb

Fig. 29 Composite Gel Electrophoresis of Tissue Protease Digests of 0.1 M NaCl Extracted Ligament Proteoglycan

Proteoglycan isolated from the bovine periodontal ligament by extraction with 0.1 M NaCl and purified by DEAE-cellulose chromatography was subjected to protease digestion followed by composite agarose-polyacrylamide gel electrophoresis as described in Fig. 27. D, cathepsin D, B, cathepsin B. TB, toluidine blue. CB, Coomassie blue. R_{bpb}, mobility relative to bromphenol blue.


A550
pH 3·6 Control
TB
0.15
0.10
0.05
D Digest TB
0.15
0.10
0.05
pH 6·0 Control TB
0.10
0.05
B Digest TB
0.10
0.05
0.2
0.4
0.6
0.8
1.0
R bpb


A550
pH 7·5 Control
TB
0.10
0.05
Elastase Digest
TB
0.10
0.05
A560
pH 6·0 Control
CB
0.10
0.05
B Digest CB
0.10
0.05
A550
Unincubated Proteoglycan
0.2
0.4
0.6
0.8
1.0
R bpb

Proteoglycan	Relative Mobility (Bromphenol Blue 1.00) Bands located with Toluidine Blue		Figure
	Control	Digest	
Skin (4 M GuCl)			
unincubated	0.73		28
cathepsin D	0.50,	0.93,	27
cathepsin B	0.61, 0.74, 0.83*	1.02, 1.44	27
elastase	0.62* 0.76, 0.85	1.02, 1.42	27
skin DS	1.05		
Periodontal Ligament (4 M GuCl)			
unincubated	0.64		27
cathepsin D	0.41	0.85	28
cathepsin B	0.50, 0.68,	0.91, 1.47*	28
elastase	0.51, 0.69	0.93, 1.47*	28
ligament GAG (18)	0.94,		
Periodontal Ligament (0.1 M NaCl)			
unincubated	0.52		29
cathepsin D	0.46	0.53, 0.60, 0.83	29
cathepsin B	0.42, 0.70	0.65 - 1.0	29
elastase	0.43	0.91	29
ligament GAG (40)	0.95		

Table 21. Gel Electrophoretic Properties of Skin and Ligament Proteoglycans and Digestion Products

Mobilities were calculated from the recordings shown in the figures. The ligament glycosaminoglycans (GAG) were those isolated by papain digestion and alcohol precipitation, 18 and 40% ethanol fractions. Skin dermatan sulphate (DS) was a pig skin preparation (Miles). * refers to very faint bands or shoulders.

Digestion of the ligament and skin proteoglycans (4 M guanidinium chloride extracted) with cathepsin D produced slightly larger fragments indicative of the presence of multiple glycosaminoglycan chains attached to peptide fragments.

The fragments produced by digestion of the ligament proteoglycan were slightly larger than those produced by digestion of the skin proteoglycans with all three enzymes, consistent with the larger size of the ligament glycosaminoglycan chains. The digestion products of the skin proteoglycan stained much more intensely with toluidine blue than did the intact proteoglycan (see for example Fig. 27 cathepsin D digest and control), suggesting that protein masking may have reduced the accessibility of toluidine blue molecules to the glycosaminoglycan chains in the intact proteoglycan. This phenomenon did not occur with the ligament proteoglycan and may reflect the lower protein content. Coomassie blue stained the intact proteoglycans very intensely, whereas the fragments produced by cathepsin D showed only very faint staining (Fig. 27, the ligament proteoglycan digest gave essentially the same scan) and those produced by cathepsin B and elastase gave no discernible staining at all (Fig. 28). This is consistent with a higher peptide to glycosaminoglycan ratio for the cathepsin D fragments. However, in view of the faint protein staining observed, it seems unlikely that the lower mobility of these fragments compared with the fragments produced by cathepsin B and elastase could be due to the presence of large peptides attached to single glycosaminoglycan chains. Coomassie blue staining of the protease digested proteoglycans showed the presence of some slower moving glycosaminoglycan-free protein species. The relative size of these proteins (presumably fragments of the proteoglycans) could not be determined from the gel electrophoresis since their charge properties were unknown but should be vastly different from that of the intact proteoglycan.

Incubation of the 4 M guanidinium chloride extracted proteoglycans in the absence of enzyme revealed an apparent aggregation of these proteoglycans indicated by the presence of a slower moving proteoglycan band (Fig. 27, pH 7.5 control) or shoulder (Fig. 28, pH 7.5 control,see also Table 21) on composite agarose polyacrylamide gel electrophoresis.

The aggregation was favoured by low pH but it also occurred at pH 7.5 (see also Pearson et al, 1978b and section 6.4). The results obtained with the skin proteoglycan were also complicated by the apparent presence of a contaminating neutral protease. Incubations at pH 7.5 in the absence of added elastase produced two bands, shown in the scan as shoulder and small peak, (Fig. 27, pH 7.5 control) that ran faster than the nonincubated control (Fig. 28). One had a relative mobility of 0.85, slightly less than that of the cathepsin D produced fragments. The other had a mobility (relative mobility 1.46) similar to the fast moving fragments produced by cathepsin B and elastase.

Digestion of the 0.1 M NaCl extracted ligament proteoglycan with tissue proteinases showed that all the lower mobility toluidine blue positive material on composite agarose polyacrylamide gel electrophoresis was of a proteoglycan nature and could be digested to molecules approaching the size of free glycosaminoglycan chains by cathepsin B and leucocytic elastase, whereas cathepsin D produced larger fragments that presumably contained a number of glycosaminoglycan chains linked to a single peptide core (Fig. 29). Similar susceptibility to the same tissue proteinases was found with cartilage proteoglycan extracted from bovine nasal septum and the fragments produced were of similar size (Roughley and Barrett, 1977).

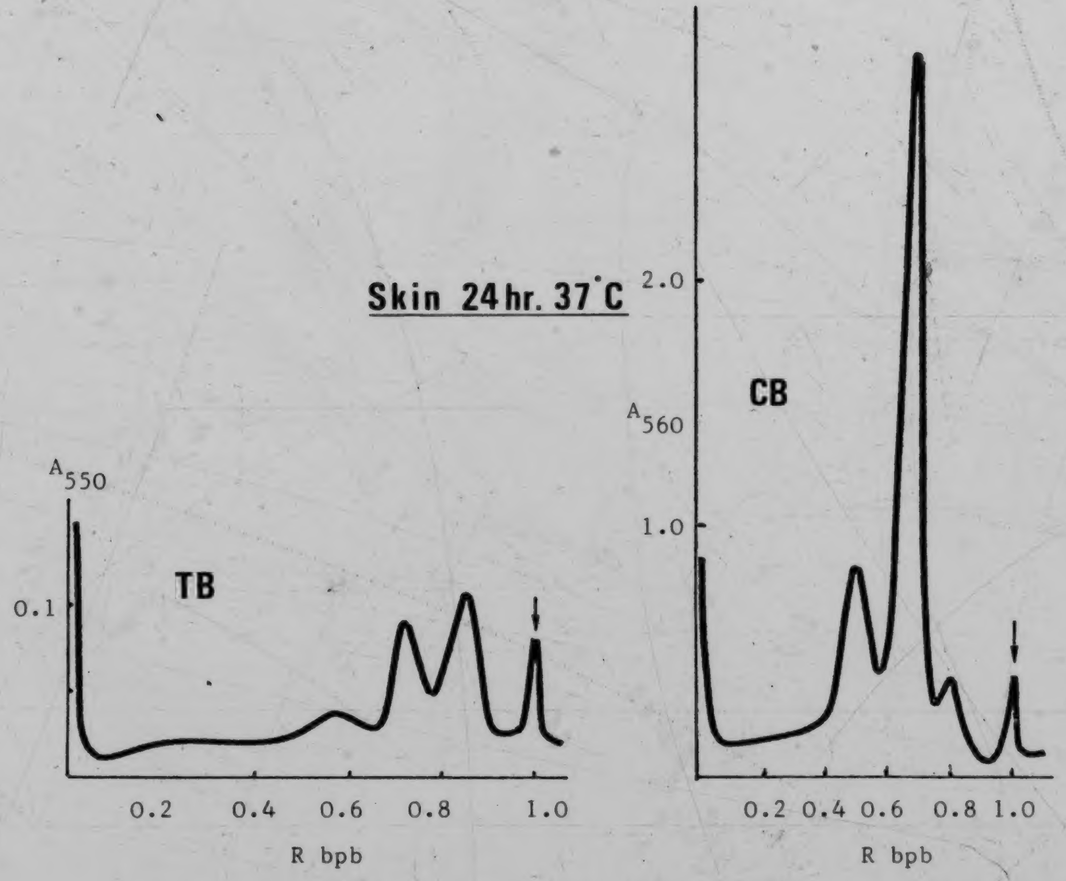
4.5 ENDOGENOUS PROTEOLYTIC ENZYME AND THE AGGREGATION BEHAVIOUR OF HIGHLY PURIFIED SKIN AND LIGAMENT PROTEOGLYCANS

In order to further investigate the apparent aggregation of the 4 M guanidinium chloride extracted proteoglycans and the possible presence of an endogenous proteolytic enzyme, highly purified proteoglycans (aliquots from the peaks obtained from Sepharose 6-B chromatography shown in Figs. 24c and 25b) were incubated at 37° C and pH 7.2 in the presence of or absence of protease inhibitors 6-aminohexanoic acid or phenylmethylsulphonyl fluoride (PMSF). The results of gel electrophoresis of the resultant material is shown in Fig. 30 and Table 22.

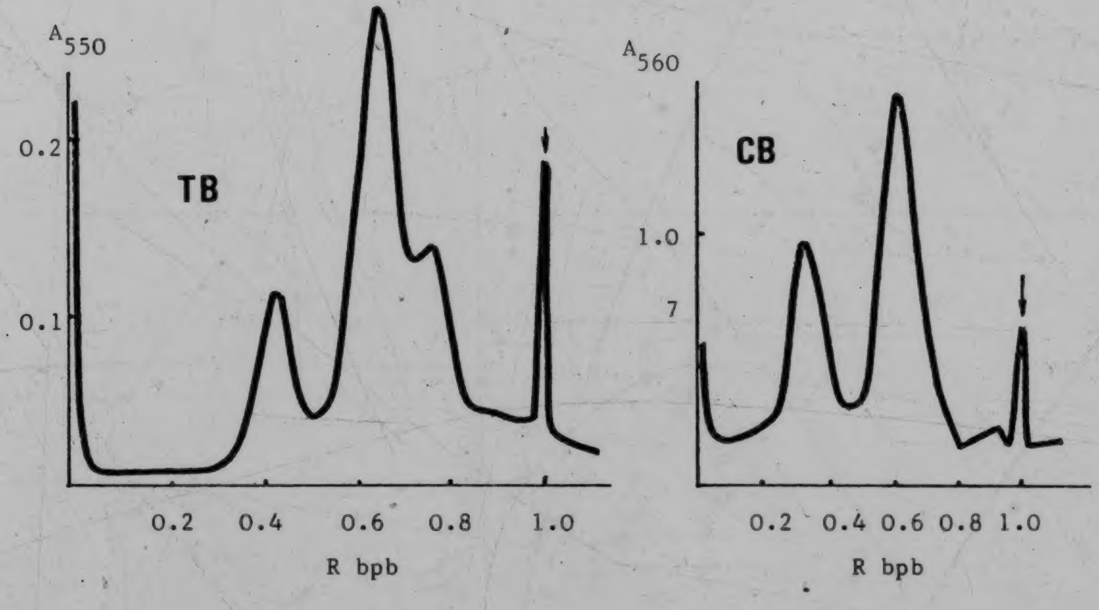
Incubation of the skin and ligament proteoglycans facilitated both their apparent aggregation and degradation. The unincubated proteoglycans ran as single sharp bands (identical to those of the less pure proteoglycans shown in Figs. 28 and 27, relative mobilities skin 0.73

Fig. 30 Gel Electrophoresis of Incubated Skin and Ligament Proteoglycans

4 M guanidinium chloride extracted skin and periodontal ligament (P.L.) proteoglycans were purified by DEAE-cellulose chromatography, density gradient centrifugation and gel chromatography (peaks shown in Figs. 24 and 25) and incubated for 24 hrs. at 37° C in 0.05 M Tris, pH 7.2. The products were then subjected to composite agarose-polyacrylamide gel electrophoresis, the bands located with Coomassie blue (CB) or toluidine blue (TB) and the gels scanned. The arrow marks the position of the marker dye, bromphenol blue. R_{bpb}, mobility relative to bromphenol blue.



Skin 24hr. 37°C



PL 24 hr. 37°C

Proteoglycan Incubation	Relative Mobility (Bromphenol blue = 1.00)		
Skin (4 M GuCl)			
nonincubated		0.73	
24 hr., 37°, pH 7.2	0.57,	0.72,	0.85
24 hr., 37°, pH 7.2 + 6-aminohexanoic acid	0.57,	0.72,	0.85
24 hr., 37°, pH 7.2 + PMSF	0.44		
Periodontal Ligament (4 M GuCl)			
nonincubated		0.64	
24 hr., 37°, pH 7.2	0.43,	0.65,	0.77
24 hr., 37°, pH 7.2 + 6-aminohexanoic acid	0.43,	0.65,	0.77
24 hr., 37°, pH 7.2 + PMSF	0.0		

Table 22. Gel Electrophoretic Properties of Incubated Skin and Ligament Proteoglycans

4 M guanidinium chloride (GuCl) extracted skin and ligament proteoglycans, purified by DEAE-cellulose chromatography, dissociative density gradient centrifugation and gel chromatography on Sepharose 6-B were incubated in 0.05 M Tris, pH 7.2 for 24 hrs. at 37° C in the absence of protease inhibitor or in the presence of 0.1 M 6-aminohexanoic acid or 1.8 mM PMSF. These samples were subjected to composite gel electrophoresis, the bands located with toluidine blue or Coomassie blue and the mobilities calculated from the recordings.

and ligament 0.64) whereas gel scans showed that after incubation a significant amount of the proteoglycan ran as a slower moving band (relative mobilities skin 0.57, ligament 0.43) that stained with both Coomassie blue and toluidine blue. Furthermore incubation gave rise to faster moving bands (relative mobilities skin 0.85, ligament 0.77) which in the case of the skin proteoglycan constituted most of the toluidine blue staining material. The faster moving band was stained more intensely with toluidine blue than with Coomassie blue and in the case of the Coomassie blue stained ligament material was visible as only a slight shoulder (Fig. 30). This suggests that the apparent degradation products contain most of the glycosaminoglycan but only a small portion of the protein of the native molecule and further supports the contention that the glycosaminoglycan chains are clustered together on a small region of protein core.

The ligament proteoglycan preparation underwent less degradation but greater apparent aggregation than the skin preparation suggesting these two phenomena may be related. This observation was supported by studies using protease inhibitors. Incubations in the presence of 1.8 mM PMSF showed only the apparent aggregation phenomenon, which it appeared to enhance, but no sign of degradation. Incubation of the skin proteoglycan in the presence of PMSF gave a single slow moving species on gel electrophoresis (relative mobility 0.44) and after similar incubation the ligament proteoglycan failed to enter the gel (Table 22). 6-aminohexanoic acid had no effect on either the aggregation or degradation phenomena and the gel electrophoresis profiles were identical to those of the proteoglycans incubated in the absence of protease inhibitors (Table 22).

Comparison of these results with those obtained with the less pure proteoglycan preparation (Fig. 27 and 28, pH 7.5 control incubations) suggest that potential degradation is enhanced with purification of the proteoglycans. However it should be noted that the earlier incubations were performed at pH 7.5 and the differences observed could be due to pH differences.

Thus, although further experiments are required to confirm the enzymic nature of the degradation, both proteoglycans appear to contain a very closely associated protease that is active at physiological pH and inhibited by PMSF, an inhibitor of serine dependant proteases, but not 6-aminohexanoic acid, an inhibitor of plasmin activity (Castellino et al, 1973). The apparent protease activity was not destroyed by the strong denaturing agents (4 M guanidinium chloride and 7 M urea) used during the preparations of the proteoglycans and must be very firmly bound to the proteoglycans, since it was not released on density gradient centrifugation in the presence of 4 M guanidinium chloride.

Both proteoglycans also appear capable of extensive aggregation although a decrease in proteoglycan mobility on gel electrophoresis could also be due to conformational changes or masking of the anionic glycosaminoglycan chains. These latter explanations, however, seem much less plausible since conformational changes would not be expected to produce such large changes in mobility and it is unlikely that the long glycosaminoglycan chains could be so extensively masked. Aggregation appears to be enhanced by low pH, incubation at 37° C and the presence of PMSF, suggesting that it is probably hydrophobic in nature and occurs between glycosaminoglycan free portions of the protein core. However the evidence to date cannot eliminate other types of binding between the protein portions of the proteoglycans.

CHAPTER 5

GLYCOSAMINOGLYCANS OF THE DEVELOPING BOVINE INCISOR PERIODONTAL LIGAMENT

In order to investigate the changes occurring in the glycosaminoglycans associated with the development of the periodontal ligament and the eruption of the bovine incisor through the mucosa into the oral cavity and finally to occlusion with the opposing bony plate, periodontal ligament was dissected from incisors of various stages of development. The progress of incisor and ligament development was examined by histology using collagen and glycosaminoglycan staining and the glycosaminoglycans were isolated and fractionated as described previously.

5.1 GROSS APPEARANCE OF THE DEVELOPING BOVINE INCISOR PERIODONTAL LIGAMENT

Periodontal ligament, obtained from bovine incisors, of various stages of development (see section 2.1), showed marked differences in gross appearance. The follicle dissected from tooth germs, prior to root development,was an almost transparent gel-like material which because of its gel-like nature was often difficult to remove from the bony crypt which surrounded the tooth germ. The first small root observed in the developing incisor was closely covered with a thin layer of tissue which, though still rather gel-like, revealed a strong fibrous attachment to the root surface upon dissection. Very little of this tissue, termed group 2 ligament, could be obtained. As the tooth developed (groups 2, 3, 4 and 5 incisors) the ligament became more difficult to remove from the tooth surface and appeared more densely fibrous. The ligament from the mature, fully occluded incisor (group 6) was the most dense and fibrous, though perhaps a little thinner than that observed with group 5 teeth. It was very firmly attached to the root surface and frequently to pieces of alveolar bone which broke away when the incisor was removed from the mandible. Pieces of attached alveolar bone were also observed, though much less frequently, with groups 4 and 5 incisors.

5.2 HISTOLOGY OF THE DEVELOPING PERIODONTAL LIGAMENT

It is generally accepted that both the periodontal ligament and cement are derived from the dental follicle which, in this dissertation, refers to the entire tissue between the developing tooth germ and the forming alveolar bone. The tissue has been considered to consist of three layers (evidence of which can be seen in Plate 2,a), an inner zone related to the tooth, an outer limiting zone separating the environment of the tooth from that of the adjacent bony crypt and between the two a predominant intermediate zone (Sicher and Bhaskar, 1972). The inner zone contains a higher concentration of cells which are believed (Ten Cate, 1969) to give rise to the fibroblasts of the periodontal ligament and cementoblasts. The predominant intermediate zone, shown in higher magnification in Plate 2,b, is a loose conglomerate of cells and fine collagen fibres in a matrix which stains strongly with alcian blue in 0.025 M $MgCl_2$ but much less intensely in 0.3 M $MgCl_2$ (Plate 2, c and d), indicative of the presence of a large concentration of hyaluronic acid. Alcian blue will stain all glycosaminoglycans in the presence of only 0.025 M $MgCl_2$, but at 0.3 M $MgCl_2$ concentrations, it will only stain the sulphated glycosaminoglycans (Scott and Doring, 1965). The outer layer, close to the bony crypt, contains thicker collagen fibres and a higher concentration of blood vessels.

The dental follicle was taken for analyses before root and ligament formation could be observed (group 1 incisors) and differences in the relative amounts of the three zones dissected or slight differences in the stage of development of this tissue may have given rise to some of the variations in analyses obtained.

A cellular cement, in association with a layer of cells, probably of epithelial, cementoblast and fibroblast origin was observed to accompany the first overt signs of root formation (group 2 incisors, Plate 2,e). Very few Sharpey's fibres were observed embedded in the cement and the collagen fibres, adjacent to the cells overlying the cement surface, ran in thick wavy bundles parallel to the root surface. Tissue dissected from the root surface of these and more developed incisors is termed ligament, in this dissertation, though the stage at

Plate 2.

a) The follicle of group 1 bovine incisors. Van Giesen stain, x 100. In, inner zone; out, outer zone; int, intermediate zone.

b) The intermediate zone of the follicle of group 1 bovine incisors. Van Giesen stain. x 400.

c) The intermediate zone of the follicle of group 1 bovine incisors. Stained with alcian blue in 0.025 M $MgCl_2$. x 400.

d) The same tissue as in c). Stained with alcian blue in 0.3 M $MgCl_2$. x 400.

e) The periodontal ligament of group 2 bovine incisors. Mid root region. Van Giesen stain. x 400.

D, dentin; C, cement; L, ligament. Apex toward bottom.

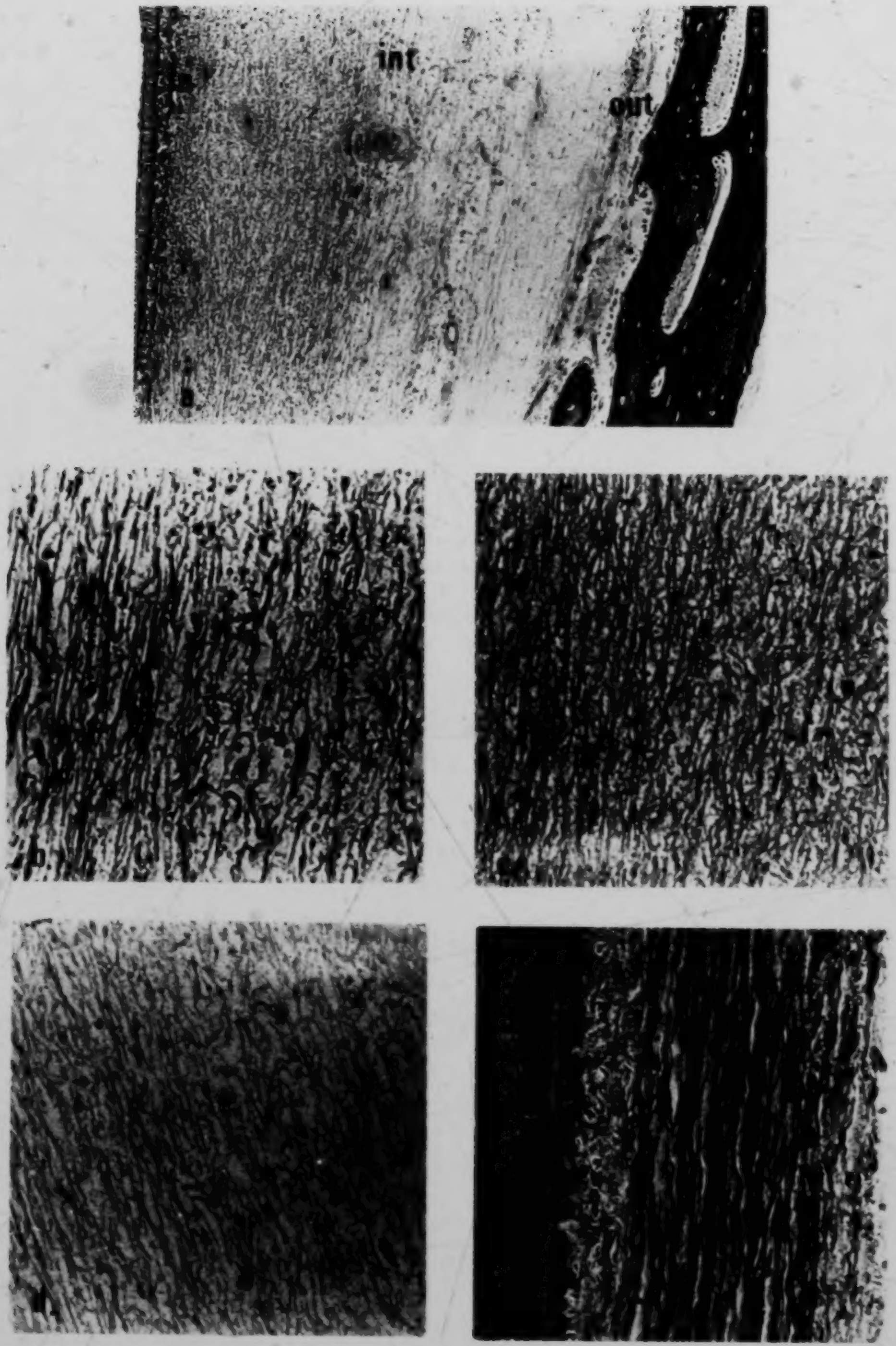

int
out
a
b

Plate 3.

a) The periodontal ligament of group 3 bovine incisors. Mid root region. Van Giesen stain. x 400.

b) The periodontal ligament of group 3 bovine incisors. Near the cemento-enamel junction. Van Giesen stain. x 400.

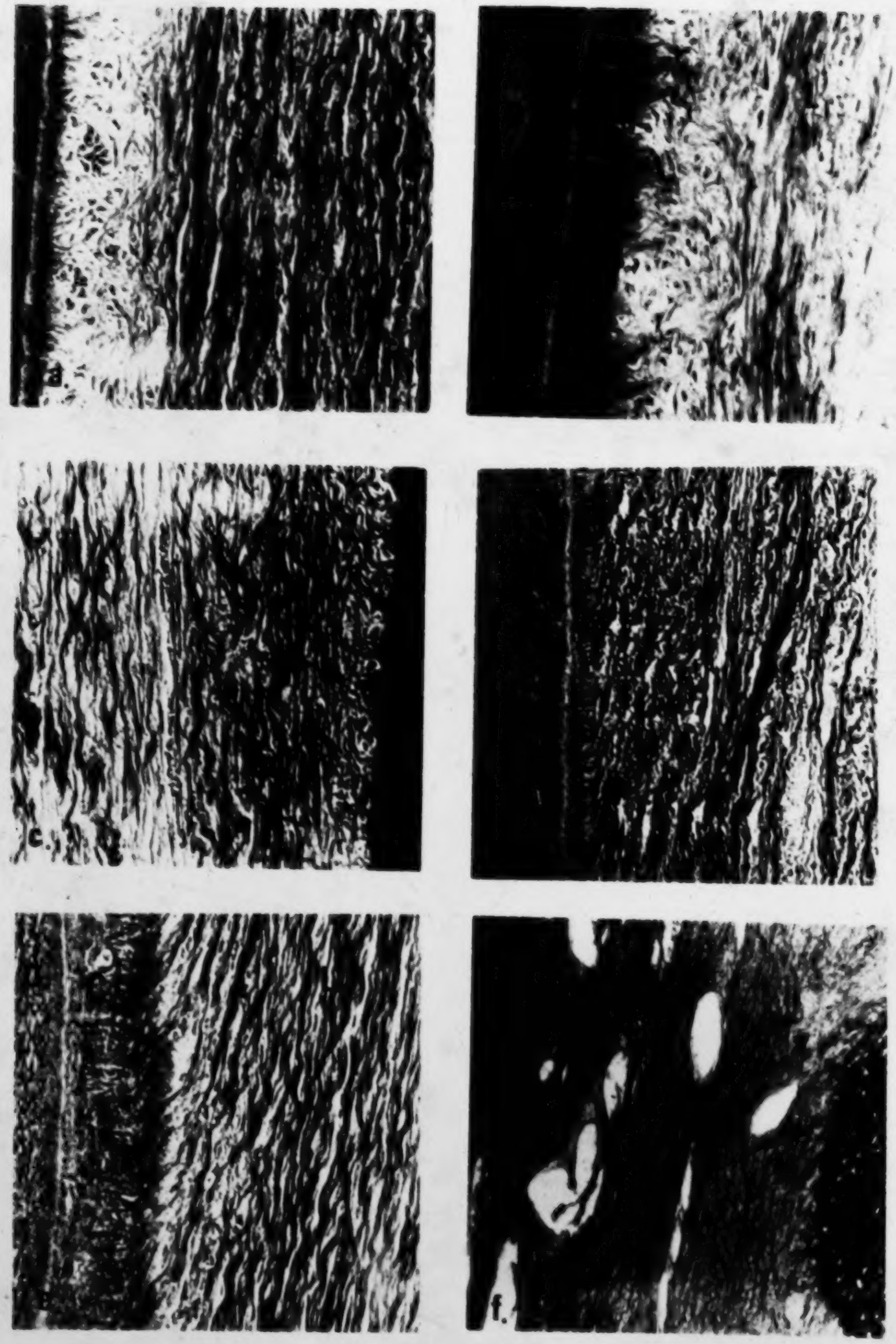
c) Group 4 bovine incisor periodontal ligament. Mid root region. Van Giesen stain. x 400.

d) Group 5 bovine incisor periodontal ligament. Toward the cemento-enamel junction. Van Giesen stain. x 250.

e) Group 6 bovine incisor periodontal ligament. Mid root region. Van Giesen stain. x 400.

f) Group 6 bovine incisor periodontal ligament. Mid root region. Van Giesen stain. x 100.

D, dentin; C, cement; L, ligament; AB, alveolar bone. Apex toward bottom.


a.
c.
f.

which the collagenous tissue investing the root surface should be called ligament remains in some doubt (see Ten Cate, 1969).

With further development of the root (group 3 incisors, Plate 3,a) a large number of Sharpey's fibres were observed. These separated the cells which had become less numerous and tended to be further from the root surface. The Sharpey's fibres were much more prominent near the cemento-enamel junction and ran, in tufts, at right angles to the root surface to form an apparently continuous network with the dense layer of collagen fibres running parallel to the root surface (Plate 3,b).

Little obvious change had occurred in the periodontal ligament at the stage of incisor development in which the crown was pushing at the mucosal surface and the root had apparently reached its full development (group 4 incisors). The number of cells close to the surface of the root had decreased and numerous Sharpey's fibres were observed embedded in the cement. Just after eruption into the oral cavity (group 5 incisors) some obliquely oriented collagen fibres were observed, for the first time, close to the cemento-enamel junction (Plate 3,d). The collagen fibres in the more apical regions of the ligament were indistinguishable from those observed in the ligament from group 3 and 4 incisors.

The ligament of the mature occluded incisor (group 6) exhibited a predominance of thick wavy collagen fibres oriented obliquely across the periodontal space. The fibres showed firm attachments to both the alveolar bone and the cement (Plate 3, e and f). The fibres embedded in the cement showed two orientations: those toward the dentin surface were oriented perpendicularly to the root surface, whereas the fibres for a small thickness near the cement surface, had an orientation similar to those in the periodontal ligament (Plate 3, e). This is in keeping with the observation that oblique orientation of the collagen fibres occurs relatively late in incisor development. Prior to this time the ligament fibres run parallel to the root surface and Sharpey's fibres embed at right angles (Plate 3, a and c). Thus a record of the orientation of fibre attachment to the cement surface may be preserved in the calcified collagen fibres entrapped in the cement.

Alcian blue staining of the periodontal ligament gave the same general appearance with all ligaments examined. Similar staining was observed in the presence of 0.025 M $MgCl_2$ and 0.3 M $MgCl_2$, except that the fibrous nature of the ligament was more prominent in the sections stained in the presence of 0.3 M $MgCl_2$ and was consistent with the observation that the sulphated glycosaminoglycans tend to have a closer association than hyaluronate with the collagenous matrix.

5.3 CHANGES IN THE COLLAGEN AND GLYCOSAMINOGLYCAN CONTENT WITH LIGAMENT DEVELOPMENT

Hydroxyproline analyses showed that the collagen content of the ligament increased markedly with development. Conversely, the hyaluronate content dropped sharply with the first signs of ligament formation but varied little with further ligament development (Fig. 31). Analysis of the chondroitin sulphate and dermatan sulphate content of the ligament by degradative procedures (sections 2.2.2.1 and 2.2.12.4) revealed little variation in the dermatan sulphate content (expressed on a collagen basis) with development. There was, however, an indication that the chondroitin sulphate content (expressed on a collagen basis) may reach a maximum in the ligament from newly erupted (group 5) incisors, but results obtained for both chondroitin sulphate and dermatan sulphate were very variable. This may have been due to variations in technique, difficulty in classification of incisors and variation between individual animals.

5.4 CHANGES IN THE ALCOHOL FRACTIONATED GLYCOSAMINOGLYCANS WITH LIGAMENT DEVELOPMENT

In order to examine the changes in ligament glycosaminoglycans without use of degradative procedures and since the sulphated glycosaminoglycans (from mature occluded incisors) were shown to consist largely of a series of copolymers (section 3.4.4), changes in ligament glycosaminoglycans with development were examined using alcohol precipitation. The 18% and 25% alcohol fractions showed a similar, approximate two fold, increase with development. The 40 and 50% alcohol fractions also showed similar changes with development. These fractions reach a maximum content in ligaments from newly erupted (group 5) incisors (Fig. 32). The greater variability observed in the 40 and 50%

Fig. 31 Changes in the Collagen and Glycosaminoglycan Composition of the Periodontal Ligament Associated with Development

Collagen composition was determined from hydroxyproline analysis of the dried ligament after hydrolysis in 6 M HCl at 105° C for 24 hrs. and is expressed as a percentage of the dry weight. Hyaluronate is the glycosaminoglycan fraction precipitated from 0.03 M NaCl with CPC and is expressed as µg uronic acid per mg hydroxyproline (µg UA in HA/mg Hyp). Dermatan sulphate and chondroitin sulphate were determined by either hyaluronidase digestion (section 2.2.2.1) or by the Di Ferrante colorimetric analyses (section 2.2.12.4) of the 0.3 M $MgCl_2$ fraction and are expressed as µg uronic acid per mg hydroxyproline. The 0.3 M $MgCl_2$ fraction is that material precipitated from 0.3 M $MgCl_2$ by CPC. Group refers to the developmental stage of the incisor from which ligament (follicle in group 1) was obtained. Group 1 refers to tooth germs before root development. Groups 2, 3 and 4 are unerupted incisors. Groups 5 and 6 are erupted incisors - group 5 prior to occlusion and group 6 fully occluded. The points are averages of a number (given in parenthesis) of determinations of individual or pooled ligaments. The bars represent standard deviations.

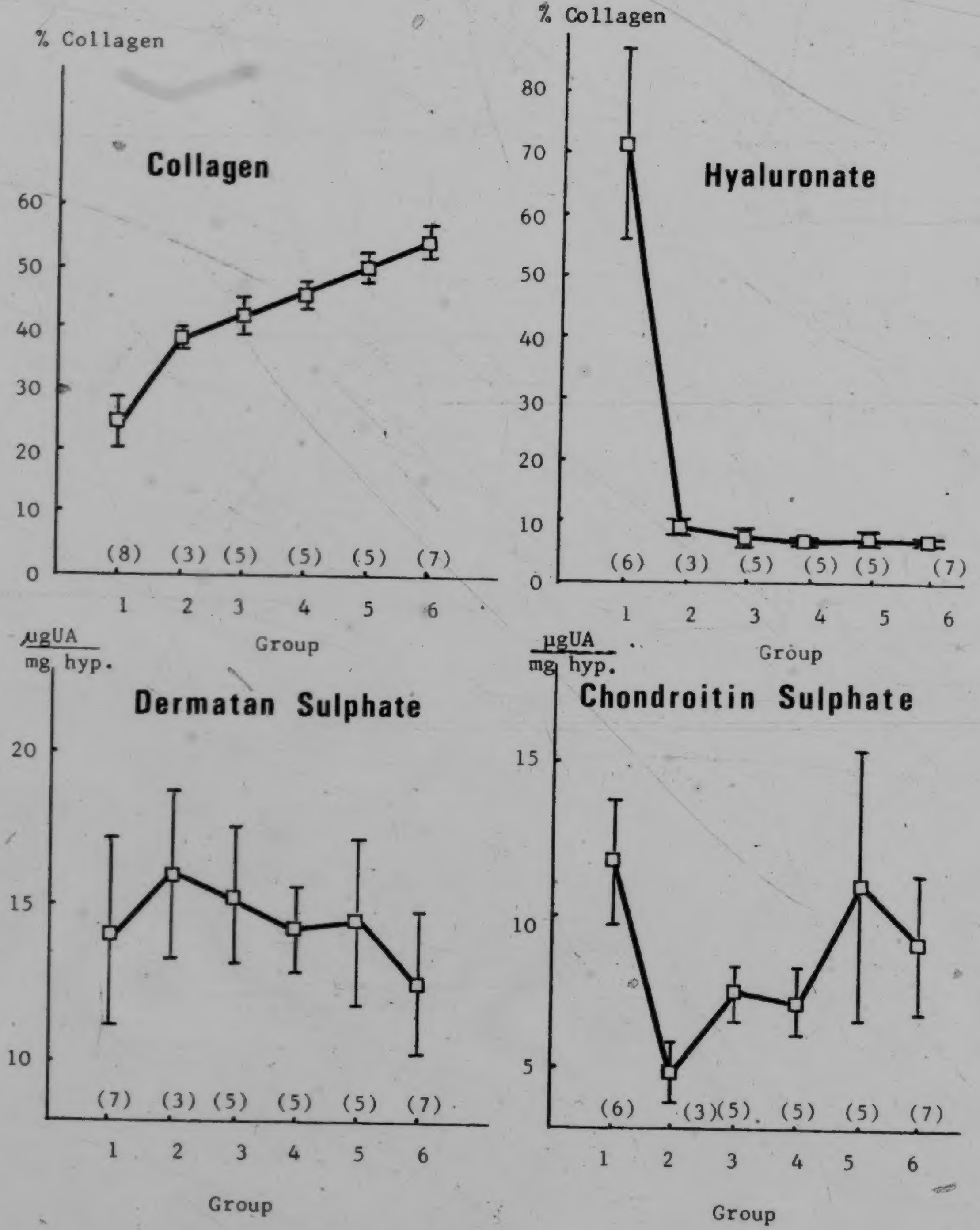
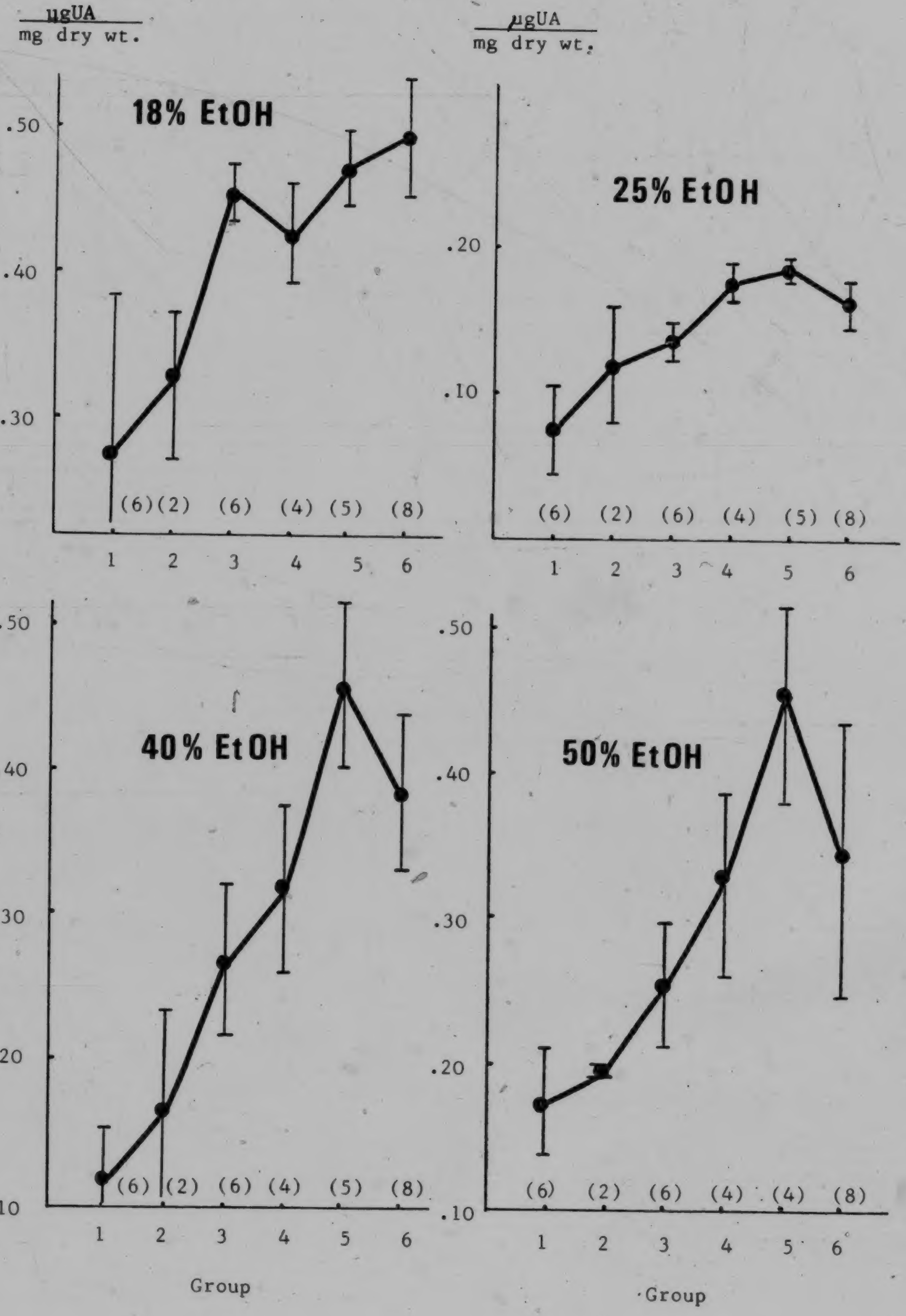

% Collagen
Collagen
60
50
40
30
20
10
0
(8) (3) (5) (5) (5) (7)
1 2 3 4 5 6
Group
% Collagen
Hyaluronate
80
70
60
50
40
30
20
10
0
(6) (3) (5) (5) (5) (7)
1 2 3 4 5 6
Group
µgUA / mg hyp.
Dermatan Sulphate
20
15
10
(7) (3) (5) (5) (5) (7)
1 2 3 4 5 6
Group
µgUA / mg hyp.
Chondroitin Sulphate
15
10
5
(6) (3)(5) (5) (5) (7)
1 2 3 4 5 6
Group

Fig. 32 Changes in the Alcohol Fractions of the Ligament Glycosaminoglycans with Tooth Development

The 0.3 M $MgCl_2$ fractions of periodontal ligaments of various stages of development were further fractionated by ethanol precipitation from calcium acetate-acetic acid solutions. The glycosaminoglycan content is expressed as µg uronic acid per mg dry weight (µg UA/mg dry wt.). The points are averages of a number (given in parenthesis) of analyses and the bars refer to standard deviations. Group, refers to the stage of tooth development.


µgUA / mg dry wt.
18% EtOH
.50
.40
.30
(6) (2) (6) (4) (5) (8)
1 2 3 4 5 6
µgUA / mg dry wt.
25% EtOH
.20
.10
(6) (2) (6) (4) (5) (8)
1 2 3 4 5 6
40% EtOH
.50
.40
.30
.20
.10
(6) (2) (6) (4) (5) (8)
1 2 3 4 5 6
Group
50% EtOH
.50
.40
.30
.20
.10
(6) (2) (6) (4) (4) (8)
1 2 3 4 5 6
Group

alcohol fractions was probably due, in part, to the greater change in ligament content of these fractions with development combined with the difficulty in classifying incisors into distinct groups.

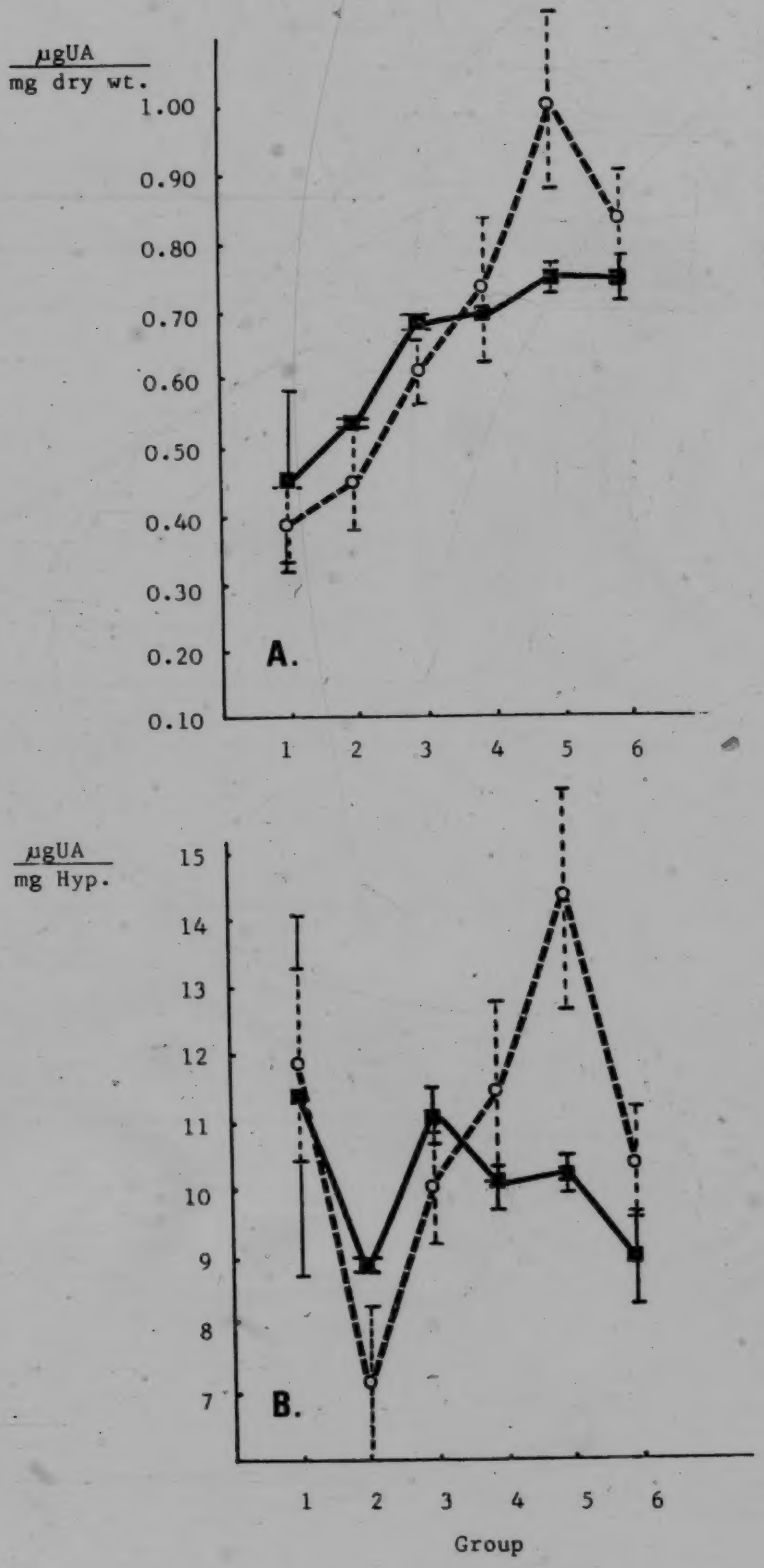
On the basis of their structural similarity (section 3.4) and parallel changes with development, the 18 and 25% alcohol fractions and 40 and 50% alcohol fractions, respectively, were combined and the results of this manipulation are shown in Fig. 33. The content of the 40 and 50% alcohol fraction increased steadily with tooth development and peaked just after eruption into the oral cavity (group 5 incisors). On the other hand the content of the 18 + 25% alcohol fraction increased with early ligament development but began to plateau well prior to tooth eruption (group 3 incisors), somewhat reminiscent of the changes observed in collagen content with ligament development (Fig. 31).

In order to more clearly observe the apparent relationship of these glycosaminoglycans to the collagen content of the ligament they were expressed on a hydroxyproline basis (Fig. 33b). Expressed in this way the 40 + 50% fraction was still observed to peak just after eruption of the incisors, but the changes observed in the 18 + 25% fraction were much less pronounced. After root formation the content (on a collagen basis) of the 18 + 25% fraction reached a maximum early in development (group 3 incisors) and then decreased slightly with further development. The two techniques of glycosaminoglycan fractionation thus give broadly similar results. The much wider variations observed in the analyses employing hyaluronidase digestion is difficult to explain, but may be due to some variability of hyaluronidase digestion or in the precipitation of partially digested glycosaminoglycan fragments.

These results suggest that the 18 and 25% alcohol fractions have similar properties and, as shown in the previous chapter, are probably linked to the same protein core. Since they appear to parallel the collagen content with development of the ligament, it is reasonable to assume that their function is closely associated with the collagen fibre network of the tissue. Similarly the 40 and 50% alcohol fractions display parallel changes with development and may be linked to the same proteoglycan. The content of the glucuronic acid rich fraction, however, reaches a definite maximum in the ligament from newly erupted incisors

Fig. 33 Changes in the Chondroitin Sulphate-like and Dermatan Sulphate-like Copolymeric Glycosaminoglycans with Ligament Development

The 18% and 25% alcohol fractions and 40% and 50% alcohol fractions, respectively, of individual glycosaminoglycan determinations were summed and plotted against the stage of incisor development. The points are averages of the analyses of individual or pooled ligaments, the number of which is the same as indicated in the previous figure. The bars refer to standard deviations. The glycosaminoglycan determinations are expressed as µg uronic acid per mg hydroxyproline (µg UA/mg hyp) and as µg uronic acid per mg dry weight (µg UA/mg dry wt.). Group refers to the stage of incisor development--○-- 40 + 50% alcohol fraction —■— 18 + 25% alcohol fraction.


µgUA
mg dry wt.
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
A.
1
2
3
4
5
6
µgUA
mg Hyp.
15
14
13
12
11
10
9
8
7
B.
1
2
3
4
5
6
Group

and does not parallel the collagen content of the ligament.

The alcohol fractions appeared homogeneous and essentially identical to the alcohol fractions obtained from the mature occluded incisor (Fig. 8 and Plate 1) on cellulose acetate electrophoresis. However, hexosamine analyses (Table 23) showed that, whereas most of the periodontal ligament samples contained only galactosamine and appeared free of contamination with hyaluronic acid or heparan sulphate, analysis of the follicle (group 1) showed that the 25, 40 and 50% alcohol fractions obtained from this tissue contained considerable levels of glucosamine. From the apparently high sulphate : hexosamine ratio in the 25 and 40% alcohol fractions, it would appear that the glucosamine is due to the presence of contaminating heparan sulphate. Conversely, the low sulphate : hexosamine ratio in the 50% alcohol fraction suggests that the glucosamine contamination in this fraction is due to hyaluronic acid. These results emphasize the necessity for rigorous checks on homogeneity before the identity of a glycosaminoglycan sample can be assumed and suggest that considerable caution should be exercised in comparing the alcohol fractions obtained from the group one tissue with those obtained from tissue of later stages of development.

Few differences in the composition of the individual glycosaminoglycan fractions were observed with ligament development (Table 23). The uronic acid content of the various fractions was similar, though a decrease in the iduronic acid content of the 40% alcohol fraction was seen with the eruption of the incisor into the oral cavity (groups 5 and 6 incisors). The proportions of 4- and 6-sulphate, determined by chondroitinase ABC digestion and subsequent paper chromatography, showed some variation, though no definite trend was apparent. Approximately 50% of the 40% alcohol fraction from the group 1 tissue was resistant to chondroitinase ABC digestion, consistent with the apparent heparan sulphate contamination of this fraction. The small amounts of chondroitinase ABC resistant material (less than 25% of the total glycosaminoglycan in each fraction) in the other fractions may be partly explained by the presence of sulphated iduronic acid residues (see section 3.4.1).

Most fractions (with the exception of the glycosaminoglycans

Table 23. Composition of the Alcohol Fractionated Glycosaminoglycans From the Developing Periodontal Ligament

Glycosaminoglycans were isolated from periodontal ligaments of various stages of development (groups 1 to 6) by papain digestion and fractionated by CPC and ethanol precipitation. Figures for the uronic acid content per mg dry weight (μg UA/mg dry wt) and uronic acid content per mg hydroxyproline (μg UA/mg hyp) are averages of a number of determinations of individual or pooled ligaments. Standard deviations are given where 4 or more determinations were performed. % iduronic acid (IdUA) refers to the amount of iduronic acid present expressed as a percentage of the total uronic acid. This was determined by the Di Ferrante technique (1971) and figures given are the averages of three or more (except group 2 where only one analysis could be performed) separate determinations. Where 4 or more determinations were performed, standard deviations are given. Sulphate was determined by the rhodizonate technique (Terho and Hartiala, 1971). 4- and 6-sulphate were analysed by the technique of Saito _et al_ (1968).

Glucosamine ($GlNH_2$) and galactosamine were determined as described in section 2.2.12.7. Total hexosamine ($HexNH_2$) was taken as the sum of the glucosamine and galactosamine. At the time the samples (*) were analysed difficulties were experienced with regard to the colour yields of hexosamine standards. In the majority of analyses the colour yields used in the calculations were determined on the same day as the sample analysis. 0.0 refers to glucosamine analyses below the limits of quantitation. In these cases the total hexosamine represents galactosamine.

We have assumed keratan sulphate to be absent from this tissue since it has only been found in cartilage, intervertebral disc and cornea (Mathews, 1975). However were keratan sulphate present it would be expected to be found in the hyaluronic acid and 0.15 M $MgCl_2$ fractions. Its absence from the ethanol fractions of the ligament (excluding group 1) is indicated by the absence of glucosamine.

Group	EtOH frn.	µg UA / mg dry wt.	µg UA / mg hyp	% IdUA	mole SO_4 / mole $HexNH_2$	$GlNH_2$ / $HexNH_2$	% 6S	% 4S
6	18	0.49 ± 0.04	6.7 ± 0.7	90 ± 5	1.1	0.0	2	98
	25	0.16 ± 0.02	2.2 ± 0.2	74 ± 3	-	-	0	100
	40	0.39 ± 0.05	5.0 ± 0.7	16 ± 3	1.1	0.0	19	81
	50	0.34 ± 0.10	5.2 ± 0.8	5 ± 3	0.95	0.0	48	52
5	18	0.47 ± 0.03	7.3 ± 0.3	85	0.99	0.0	3	97
	25	0.19 ± 0.01	2.9 ± 0.2	72	-	-	11	89
	40	0.46 ± 0.06	7.3 ± 0.9	18	1.1	0.0	33	67
	50	0.45 ± 0.07	7.1 ± 0.9	4	0.8	0.0	56	44
4	18	0.43 ± 0.04	7.1 ± 0.5	87	1.0*	0.0	4	96
	25	0.18 ± 0.01	3.0 ± 0.2	74	1.1*	0.0	10	90
	40	0.32 ± 0.06	5.5	26	0.96*	0.0	22	78
	50	0.32 ± 0.06	5.7 ± 0.7	4	0.58*	0.0	39	61
3	18	0.46 ± 0.02	8.7 ± 0.1	91	0.88*	0.0	18	82
	25	0.14 ± 0.01	2.5 ± 0.3	75	-	-	17	83
	40	0.27 ± 0.05	5.0 ± 0.9	21	0.72*	0.2	34	66
	50	0.25 ± 0.04	4.9 ± 0.8	7	0.88*	0.0	53	47
2	18	0.32	6.3	90				
	25	0.12	2.4	73				
	40	0.16	3.2	35				
	50	0.20	3.8	5				
1	18	0.28 ± 0.15	9.1 ± 2.1	95 ± 5	0.95*	0.0	18	82
	25	0.07 ± 0.03	2.4 ± 0.7	67 ± 10	2.0*	0.2	18	82
	40	0.12 ± 0.04	3.3 ± 1.0	19 ± 4	1.9*	0.6	46	54
	50	0.17 ± 0.04	8.4 ± 1.4	5	0.53*	0.2	47	53

Table 23. Composition of the Alcohol Fractionated Glycosaminoglycans From the Developing Periodontal Ligament

isolated from group 1 follicle) contain approximately equimolar sulphate and hexosamine. The slightly lower sulphate content of the 40% alcohol fraction isolated from the group 3 ligaments may be explained by the presence of a small amount of hyaluronic acid indicated by the presence of some glucosamine (Table 23). The 50% alcohol fraction of the group 4 ligament appears to be significantly undersulphated. However these analyses were not consistent with the results obtained from chondroitinase ABC digestion. The non-sulphated unsaturated disaccharide (Δdi 0-S) could not be detected on paper chromatography even though greater than 80% of this glycosaminoglycan fraction was digested with chondroitinase ABC. At the present time this anomaly cannot be explained but may be due to inadequate separation of the Δdi 4-S and Δdi 0-S unsaturated disaccharides.

CHAPTER 6

DISCUSSION

6.1 PERIODONTAL LIGAMENT GLYCOSAMINOGLYCANS

Using CPC-precipitation combined with hyaluronidase digestion or the colorimetric analyses for dermatan sulphate I have shown that the periodontal ligament of the mature bovine incisor contains 0.67% (dry weight) glycosaminoglycan which is made up of 31% chondroitin sulphate type glycosaminoglycan, 43% dermatan sulphate type glycosaminoglycan, 24% hyaluronic acid, a small proportion of material tentatively identified as containing heparan sulphate and possibly some undersulphated galactosaminoglycan. These results agree remarkably well with the analysis of bovine molar periodontal ligament reported by Pearson et al (1975) using similar techniques. Although they did not determine the glycosaminoglycan content they showed the same types of glycosaminoglycan were present in almost precisely the same proportions. Paunio (1969) had previously shown that the human periodontal ligament contained sulphated galactosaminoglycans and also hyaluronic acid although the proportion of hyaluronic acid reported in this material was much higher. Munemoto et al (1970) also reported a similar glycosaminoglycan composition of the bovine molar periodontal ligament although they reported the presence of approximately twice as much chondroitin sulphate type as dermatan sulphate type glycosaminoglycan.

These estimations, however, relied upon degradative techniques to distinguish the dermatan sulphate from the chondroitin sulphate polymers and thus give little idea of the content and structure of the native galactosaminoglycans.

6.1.1 Copolymeric Nature of the Periodontal Ligament Galactosaminoglycans

A wide range of copolymeric structures containing both L-iduronic acid and D-glucuronic acid have been isolated from a variety of tissues (Fransson, 1968, Fransson and Rodén, 1967a, Fransson and Havsmark, 1970, Fransson et al, 1970, Habuchi et al, 1973 and Inoue and Iwasaki, 1976).

The proportion of glucuronic acid varies from only a few percent in glycosaminoglycans like those from skin (Fransson and Rodén, 1967a) to constitute the majority of the uronic acid in glycosaminoglycans like those isolated from umbilical cord (Inoue and Iwasaki, 1976), meniscal cartilage (Habuchi et al, 1973) and aorta (Fransson and Havsmark, 1970). Furthermore a wide range of copolymers frequently occurs in a single tissue (Inoue and Iwasaki, 1976, Habuchi et al, 1973 and Fransson and Havsmark, 1970).

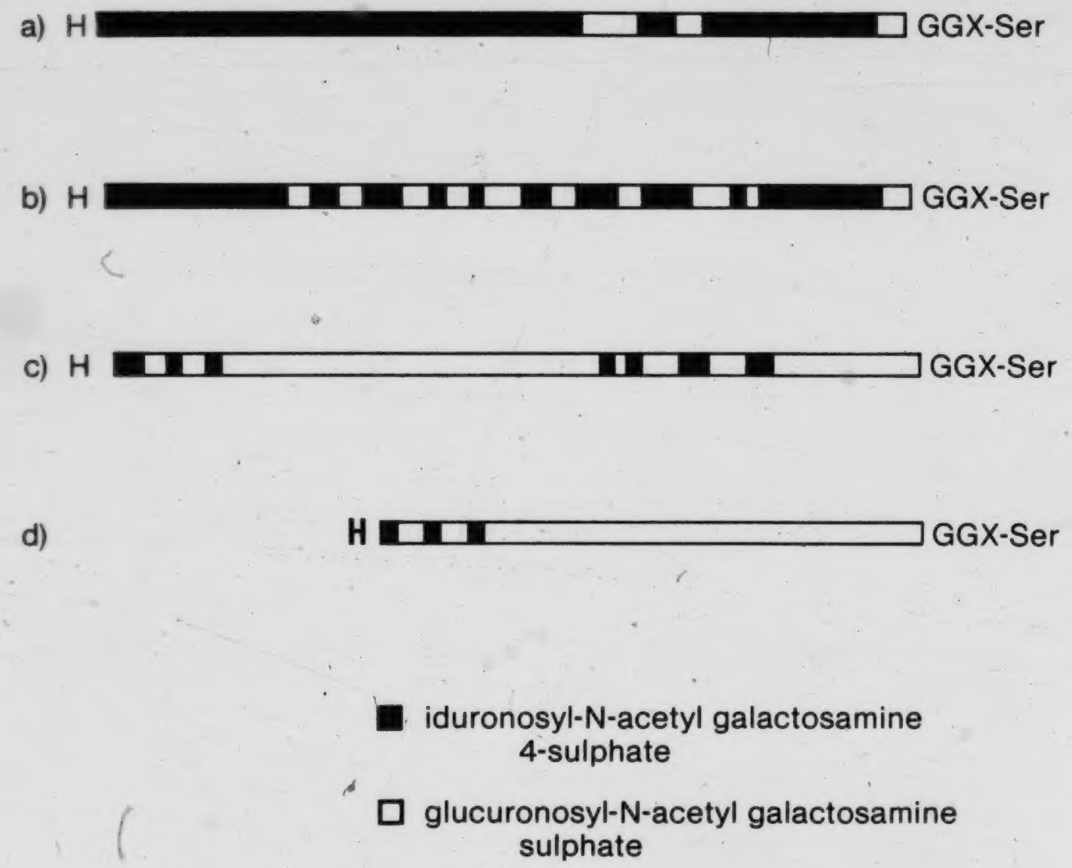
Ethanol fractionation resulted in resolution of the periodontal ligament galactosaminoglycans into a series of variants of differing iduronic acid content. These can be divided into approximately equal proportions of glucuronic acid rich (40 and 50% alcohol fractions) and iduronic acid rich (18 and 25% alcohol fractions) polymers. Hyaluronidase digestion and periodate-alkali cleavage established that the fractions contained a predominance of copolymeric glycosaminoglycans that varied in the proportions and distribution of iduronic and glucuronic acid, though the experiments performed could not eliminate the presence of small amounts of dermatan sulphate and chondroitin sulphate homopolymers. On the basis of the results obtained a number of hypothetical models can be proposed for the prevalent hybrid structures of the periodontal ligament galactosaminoglycans (Fig. 34).

Hyaluronidase digestion of copolymeric glycosaminoglycan represented by model a) would yield predominantly large oligosaccharides containing few glucuronic acid residues, a much smaller amount of intermediate sized oligosaccharides containing approximately equal proportions of glucuronic and iduronic acid and a similar number of smaller oligosaccharides containing few iduronic acid residues. Periodate oxidation and alkali cleavage would produce extensive degradation to small and intermediate sized oligosaccharides. The 18% alcohol fraction followed this pattern of degradation (Fig. 15 and Table 12) and probably has a hybrid structure similar to that in model a).

The 25% alcohol fraction gave a degradation pattern similar to the 18% alcohol fraction except the proportion of intermediate sized

Fig. 34 Schematic Models of Some Possible Hybrid Structures of the Periodontal Ligament Galactosaminoglycans

The iduronosyl-N-acetylgalactosamine 4-sulphate repeating disaccharide residues are represented by solid lines. The blank areas represent glucuronosyl-N-acetylgalactosamine sulphate repeating disaccharide residues (2mm = 1 disaccharide). H, the non reducing terminal; GGX, the neutral trisaccharide galactosylgalactosylxylose of the linkage region. The lengths of the respective regions are very approximate and are calculated from the molecular size of the polysaccharides as well as the degradation patterns.


a) H
GGX-Ser
b) H
GGX-Ser
c) H
GGX-Ser
d) H
GGX-Ser
■ iduronosyl-N-acetyl galactosamine 4-sulphate
□ glucuronosyl-N-acetyl galactosamine sulphate

(retarded on Sephadex G-50) oligosaccharides containing interspersed glucuronic and iduronic acid containing disaccharides was much greater. Similarly the extent of periodate oxidation - alkali cleavage was reduced (Fig. 15). This degradation pattern is compatible with the hybrid structure proposed in Model b).

The 40 and 50% alcohol fractions contained much more glucuronic acid and as might be expected, hyaluronidase degradation was much more extensive and periodate-alkali cleavage considerably reduced. The model hybrid structures proposed for the 40 and 50% alcohol fractions are models c) and d) respectively. The models are similar except that c) contains more iduronic acid residues, some of which are located in more central regions of the polysaccharide chain, whereas model d) contains iduronic acid residues only at the non-reducing end of the chain. Model d) would exhibit only a small change in molecular size on periodate oxidation-alkali cleavage similar to that observed with the 50% alcohol fraction. The small amount of material excluded from Sephadex G-50 on hyaluronidase digestion of the 40 and 50% alcohol fractions (Fig. 15) is compatible with the models proposed in c) and d) if we realize that hyaluronidase is not a very efficient enzyme and, as demonstrated by the presence of large quantities of hexa and octasaccharides after hyaluronidase digestion of chondroitin sulphate (Ludoweig et al, 1961), does not cleave all the hyaluronidase susceptible linkages. Thus a small portion of large oligosaccharides could be expected to arise from the non-reducing ends of the chains proposed in models c) and d). The corresponding fractions isolated from the ligament galactosaminoglycans contained approximately equal proportions of glucuronic and iduronic acid, compatible with the models proposed.

Although these models are consistent with the uronic acid content and the results obtained from hyaluronidase digestion and periodate oxidation - alkali cleavage of the 18, 25, 40 and 50% alcohol fractions of the ligament galactosaminoglycans it should be emphasized that each fraction probably contains a large number of different variants and the proposed models serve to illustrate the probable average type of hybrid structure of the respective fractions.

The type of hybrid structure proposed is similar to those suggested for a number of copolymeric galactosaminoglycan preparations isolated from a variety of tissues (Fransson and Rodén, 1967a and b, Fransson and Havsmark, 1970, Fransson and Malmström, 1971 and Fransson et al, 1974). All the structures reported had a number of general features in common. A major fraction of both the D-glucuronic and the L-iduronic acid units occur in blocks composed of varying numbers of only glucuronosyl-N-acetylgalactosamine or only iduronosyl-N-acetylgalactosamine repeating disaccharide units, respectively and the relative proportions of the two types of blocks has been found to vary considerably (Habuchi et al, 1973). Although each kind of block structure was found to occur anywhere along the polysaccharide chain, there was generally an over-representation of D-glucuronic acid units in the immediate vicinity of the carbohydrate-protein linkage. In the transition zones between more extended blocks, glucuronic acid - and iduronic acid - containing sequences alternated at shorter intervals.

The periodontal ligament hybrid galactosaminoglycans all contained approximately equimolar proportions of galactosamine and sulphate, although the position of sulphation varied considerably (Table 10). The fractions containing few glucuronic acid residues (18 and 25% alcohol fractions) contained 4-sulphated galactosamine residues almost exclusively, whereas the fractions containing few iduronic acid residues (the 40 and 50% alcohol fractions) contained N-acetylgalactosamine residues sulphated in either the 4- or 6- position. The 50% alcohol fraction contained approximately equal proportions of 4- and 6-sulphated N-acetylgalactosamine residues. These results concur with the general tendency toward the prevalence of L-iduronosyl-N-acetylgalactosamine 4-sulphate and D-glucuronosyl-N-acetylgalactosamine 6-sulphate units (Fransson and Havsmark, 1970, and Habuchi et al, 1973). Nevertheless a large proportion of the glucuronic acid residues in the ligament copolymers must occur linked to N-acetylgalactosamine 4-sulphate.

Some understanding of the structural features of these copolymeric galactosaminoglycans may be gained from studies of the mechanism of cellular synthesis. Recent results using a particulate subcellular fraction from cultured skin fibroblasts (Malmström et al, 1975) indicate that the incorporation of L-iduronic acid units into dermatan sulphate occurs in a closely similar manner to that found in heparin biosynthesis (section 1.3.1.4), involving C5 inversion of D-glucuronic acid residues at the polymer level. In dermatan sulphate, as in heparin, the process was strongly promoted by concomitant sulphation of the polymer. In the sulphated product about one-third of the uronic acid units were L-iduronic acid, a major portion of which occurred in block structures composed of alternating L-iduronic acid and N-acetylgalactosamine 4-sulphate residues. However, the two processes display certain dissimilarities. In the case of heparin, no L-iduronic acid could be detected in products formed in the absence of sulphation (Höök et al, 1975), whereas the galactosaminoglycan produced under similar conditions by the fibroblast system contained isolated (i.e. surrounded by glucuronic acid - containing repeating disaccharide units) iduronic acid residues, amounting to 5-10% of the total uronic acid. However subsequent sulphation of the nonsulphated galactosaminoglycan in the fibroblast particulate system increased the proportion of iduronic acid 3 to 5 fold (Malmström et al, 1975).

Sulphation, and sulphation at C4 in particular, of N-acetylgalactosamine, seems to be intimately associated with the C5 inversion of uronic acid residues. There is some evidence to suggest, at least in heparin biosynthesis, that the sulphotransferase(s) and C5 inversion enzyme may be tightly bound to a common membrane structure in such a way as to render the two events (sulphation and uronic acid inversion) inseparable or tightly coupled (Lindahl, 1976). However in cells that synthesize dermatan sulphate the membrane bound enzyme complex may be less firmly associated and separation of the two processes either by distance or time may produce a modulation in the iduronic acid content of the type observed with periodontal ligament copolymers.

The uronic acid composition of this type of polysaccharide may also be influenced by the presence of a so called reverse epimerase. Fransson et al (1973) showed that fibroblasts in culture synthesized and secreted a product which contained very little D-glucuronic acid. Some of the L-iduronic acid moieties of this polymer were subsequently transformed to D-glucuronic acid residues without cleavage of the dermatan sulphate chains. The epimerization reaction apparently took place extracellularly and was catalysed by an enzyme derived from the fibroblasts. Incubation of a labelled dermatan sulphate preparation with the cell medium (medium in which the cells had been grown for 3 days) resulted in an increase in the glucuronate content of the polymer from a few residues to 20% of the total uronic acid.

However our knowledge of the mechanism of biosynthesis of the iduronic acid containing galactosaminoglycans is still very limited. The influence of the proteoglycan nature of these galactosaminoglycans on their synthesis and factors that control the relative proportion of iduronic acid and glucuronic acid are still largely unknown.

The recent work of Fransson (1976), describing the interchain binding between copolymeric galactosaminoglycans and other glycosaminoglycans, has ascribed a functional significance to the copolymeric nature of these galactosaminoglycans. The copolymer was attached to agarose gels and various glycosaminoglycans were then chromatographed on this material. When the same kind of copolymer was chromatographed on the gel it bound at 0.15 M NaCl but could be eluted with 1 M urea or 0.5 M guanidinium chloride or 0.4 M NaCl. The most pronounced interaction occurred if the copolymer contained approximately equal amounts of iduronosyl- and glucuronosyl-N-acetylgalactosamine sulphate. However a certain interaction occurred between the copolymer and a homopolymer of chondroitin 4-sulphate as well as with heparan sulphate and heparin. No interaction was observed with chondroitin 6-sulphate, hyaluronic acid or keratan sulphate.

6.1.2 The Molecular Weight of the Ligament Galactosaminoglycans and Their Behaviour on Gel Chromatography

The results obtained from end-group analysis of the ligament and skin glycosaminoglycans gave number average molecular weights of 30.8×10^3, 29.8×10^3, 21.0×10^3, and 18.2×10^3 for the 18, 40, and 50% alcohol fractions isolated from the ligament proteoglycans and the 25% alcohol fraction isolated from the skin proteoglycan, respectively.

Gel chromatography of the end-labelled glycosaminoglycans on Sephadex G-200 enabled their separation into a large number of essentially monodisperse fractions. The relationship of the partition

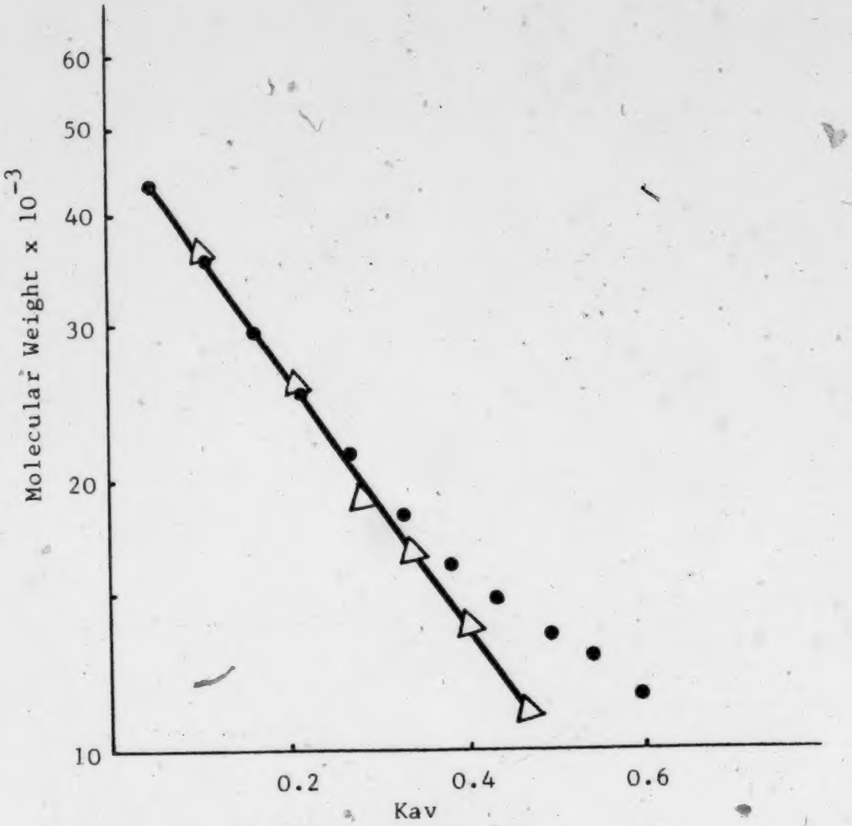


Fig. 35 The Molecular Weights of Two Series of Chondroitin Sulphate Fractions as a Function of Their Kav

The partition coefficient (Kav) between Sephadex G-200 and buffer of the chondroitin sulphate fractions (●) isolated from bovine nasal cartilage proteoglycan by alkaline cleavage, and reduction with NaB^3H_4 and a series of chondroitin 4-sulphate polymers taken from the data reported by Wasteson (1971) (▷) are plotted against their respective molecular weights.

coefficient between the buffer and the gel resin to the molecular weight of the individual fractions from gel chromatography, gave some idea of the relative molecular conformations of the glycosaminoglycans. These studies showed that all glycosaminoglycan fractions examined conformed to very similar straight line relationships (Fig. 12), suggesting that they all had similar conformations in 0.2 M NaCl, 0.02 M imidazole buffer at pH 6.8. Moreover, if the partition coefficients on Sephadex G-200 and the respective molecular weights (determined by equilibrium sedimentation) of a homologous series of chondroitin 4-sulphate polymers reported by Wasteson (1971) are plotted in the same way, the two sets of data are almost precisely superimposed (Fig. 35). Although Constantopoulos et al (1969) calibrated a Sephadex G-200 column with chondroitin 4-sulphate standards of known molecular weight and found that a dermatan sulphate sample of known molecular weight fell on the same calibration line, a thorough comparison of the behaviour of the different glycosaminoglycans on gel chromatography had not been reported before the present work.

It should be emphasized here that although the glycosaminoglycans of the isolated proteoglycans were not thoroughly characterized, the proteoglycans extracted represent over 90% of the uronic acid of the periodontal ligament and hence, their glycosaminoglycan fractions would be expected to have the same structure as the equivalent fractions isolated from the whole tissue. Thus the present studies show that five galactosaminoglycan preparations (40% alcohol fraction isolated from cartilage and the 18, 40 and 50% alcohol fractions isolated from the periodontal ligament and the 25% alcohol fraction isolated from skin), exhibiting broad variations in the relative proportions of iduronic acid and glucuronic acid and galactosamine 4-sulphate and 6-sulphate and ranging in molecular weight from 18,000 to 30,800, have essentially the same elution behaviour on Sephadex G-200 and thus similar chain conformations. These studies further suggest that gel chromatography columns can be calibrated with any series of galactosaminoglycans of known molecular weight, such as those produced by the end-labelling of chondroitin sulphate from cartilage proteoglycan, to determine the molecular weights of all types of galactosaminoglycans.

The $\bar{M}w/\bar{M}n$ ratio determined for the cartilage chondroitin sulphate (1.127) was very similar to those previously reported (1.09, Hopwood and Robinson, 1973 and 1.22-1.28, Wasteson, 1969 and 1971) and suggests that these glycosaminoglycans are slightly more polydisperse than the skin and ligament galactosaminoglycans. ($\bar{M}w/\bar{M}n$ 1.004 - 1.10). However Hopwood and Robinson (1973) have reported the presence of two distinct pools of chondroitin sulphate chains in bovine nasal septum cartilage with $\bar{M}w/\bar{M}n$ ratios of 1.03 and 1.04 and hence rather low polydispersity similar to those determined for the majority of the ligament and skin glycosaminoglycans.

The 18% alcohol fraction from the 4 M guanidinium chloride extracted ligament proteoglycan showed strikingly different polydispersity from the other glycosaminoglycans. The $\bar{M}w/\bar{M}n$ ratio was very close to 1.0 and gel chromatography of Sephadex G-200 gave a very sharp peak suggestive of a monodisperse fraction. I have been unable to find any other reports of a monodisperse glycosaminoglycan, isolated either from a tissue or purified proteoglycan. Polydispersity has been thought to be one of the characteristics of glycosaminoglycans (Bettelheim, 1970) and has been suggested to arise due to the lack of a chain termination mechanism (Rodén and Schwartz, 1975). Hopwood and Robinson (1973) suggested that the size of the chondroitin sulphate chains synthesized in bovine nasal cartilage could be regulated by the rate at which the initiator molecule is transported along the endoplasmic reticulum past a complex array of membrane-bound glycosyltransferases (Rodén and Schwartz, 1975). Though this could conceivably be the case for most of the glycosaminoglycans examined, monodispersity of the 18% alcohol fraction of the ligament proteoglycan suggests that a specific chain termination mechanism must occur but may be peculiar to this proteoglycan species.

6.2 THE PROTEOGLYCANS OF THE PERIODONTAL LIGAMENT

The sequential extraction procedure used in these experiments was effective in extracting the bulk (greater than 90%) of the proteoglycans (as measured by uronic acid and the Di Ferrante dermatan sulphate analyses) of bovine skin and periodontal ligament. The majority of the dermatan sulphate containing proteoglycan was extracted with 4 M guanidinium chloride, although a considerable proportion (17.1%) could be extracted from the periodontal ligament with 0.1 M NaCl. Analyses of the ligament glycosaminoglycans showed that a large proportion of the dermatan sulphate containing material in the low salt extracts could be due to the presence of copolymers containing small proportions of iduronic acid. However the relative amount of dermatan sulphate suggested that some iduronic acid rich glycosaminoglycan containing material may have been present. These observations were supported by subsequent purification of the proteoglycans and confirm the previous observations of Pearson et al, (1975) that approximately a quarter of the total dermatan sulphate from bovine molar periodontal ligament could be extracted with 0.15 M NaCl.

Although the high yields of proteoglycan were unexpected when the initial experiments were performed, several laboratories have since reported equally efficient extraction of proteoglycans from bovine cornea and sclera (Antonopoulos et al, 1974), bovine aorta (Antonopoulos et al, 1974, Ehrlich et al, 1975 and Eisenstein et al, 1975) and from newborn calf dermis (Yamanishi and Sato, 1976).

Efficient purification of the proteoglycans was achieved by DEAE-cellulose chromatography and density gradient centrifugation. The 2 M NaCl fraction from DEAE-cellulose chromatography was largely free of collagen and further purification by density gradient centrifugation separated most of the non proteoglycan protein from the proteoglycan material. However it may be noted that gel chromatography on Sepharose 6-B appears to be as efficient as density gradient centrifugation in purifying the 4 M guanidinium chloride extracted proteoglycans, since the proteoglycan fraction isolated from gel chromatography (Figs.24 and 25) had precisely the same elution position and protein and uronic acid content regardless of whether density gradient centrifugation had been performed.

The extraction and purification conditions employed were mild and high shear forces and extremes of pH were avoided to minimize the risk of degradation of the proteoglycans. Furthermore extractions were performed at pH 7.6 in order to inactivate any cathepsin D likely to be present and extraction solutions contained a cocktail of protease inhibitors to inactivate tissue proteinase active at pH 7.6.

6.2.1 The Chondroitin Sulphate Proteoglycans

Purification of the 0.1 M NaCl extract of the periodontal ligament showed it to contain a predominance of proteoglycans of large molecular size, high buoyant density and low protein content. The major glycosaminoglycans isolated from these proteoglycans belonged to the chondroitin sulphate family, though they contained small proportions of iduronic acid. The predominant proteoglycan in the 0.1 M NaCl extract of the periodontal ligament resembles the chondroitin sulphate proteoglycans isolated from the cornea (Axelsson and Heinegård, 1975) and the aorta (Kresse et al, 1971 and Antonopoulos et al, 1974). These proteoglycans appear to have similar elution volumes on Sepharose 2-B and are composed of glycosaminoglycans that contain similar small proportions of iduronic acid (20% and 10% of the total uronic acid in the aorta and corneal preparations respectively). Though this type of proteoglycan tends to have slightly higher protein contents and differs slightly in glycosaminoglycan composition, it resembles the proteoglycan subunit isolated from cartilage.

The proteoglycans extracted from the periodontal ligament with 0.1 M NaCl showed a similar susceptibility to tissue proteinases as those extracted from bovine nasal cartilage. Digestion of the ligament proteoglycan with leukocytic elastase or cathepsin B produced some fragments with the mobility of free glycosaminoglycan chains on composite agarose polyacrylamide gel electrophoresis whereas cathepsin D produced no single chains but only relatively large fragments that presumably contained a number of glycosaminoglycan chains linked to a single peptide core (Fig. 29). Cartilage proteoglycans gave very similar size digestion products though elastase produced slightly larger fragments and only cathepsin B (at pH 5.0 cf. pH 6.0 here) produced single chains (Roughley and Barrett, 1977).

The recent discovery that proteoglycans with the ability to aggregate with hyaluronic acid can be extracted from the aorta has provided concrete evidence that proteoglycans with most of the characteristics of cartilage proteoglycans can be found in other tissues. Gardell (1978 personal communication) extracted proteoglycans from the aorta with 4 M guanidinium chloride, taking rigorous precautions to avoid proteolytic cleavage due to endogenous protease activity. The proteoglycans, purified by dissociative density gradient centrifugation, were shown to be larger than the subunit extracted from bovine nasal septum cartilage and had the ability to aggregate with hyaluronic acid. Furthermore he showed that the proteoglycan, if prepared in an associative density gradient, contained molecules of immunological identity with the link proteins and the hyaluronate binding region of bovine nasal septum proteoglycan.

The apparent degradation of the proteoglycans previously prepared from these tissues presumably reflects the action of endogenous proteolytic activity occurring either within the tissue or during extraction and purification of the proteoglycans. However some degradation also appears to occur during purification by DEAE-cellulose chromatography in 7 M urea. Although the mechanism of degradation is unexplained, proteoglycans from the aorta when purified in this way were smaller and lacked the ability to aggregate with hyaluronic acid (Gardell, 1978, personal communication). These findings suggest that the proteoglycan in the 0.1 M NaCl extract of the periodontal ligament may be partially degraded either due to protease activity occurring within the tissue or during purification of the proteoglycan by DEAE-cellulose chromatography in 7 M urea. Partial degradation of the proteoglycan may also explain some of the heterogeneity observed in this fraction. In accord with these proposals amino acid analysis of the ligament proteoglycan preparation showed some similarity with the non-aggregating proteoglycan extracted from laryngeal cartilage with 0.15 M NaCl (Hardingham and Muir, 1974, Table 19). It has been suggested that this proteoglycan arises as a result of enzymic cleavage of the hyaluronic acid binding region of the predominant cartilage proteoglycan (Hardingham _et al_, 1976).

These studies suggest that the chondroitin sulphate proteoglycans isolated from a number of connective tissues may have been degraded either by enzymic or chemical action during extraction and purification and that proteoglycans with the characteristics (including the ability to aggregate with hyaluronic acid) of cartilage type proteoglycans may be common to many connective tissues in the body, including the periodontal ligament.

6.2.2 Dermatan Sulphate Proteoglycan

Purification of the 4 M guanidinium chloride extracts from skin and periodontal ligament showed that both extracts contained a single predominant species of proteoglycan. The ligament proteoglycan was slightly larger but otherwise very similar to the skin preparation and ran as a sharp peak (buoyant density 1.45 gm/ml) on density gradient centrifugation. Subsequent gel chromatography on Sepharose 6-B gave a single predominant peak (Kav, 0.36) that contained 47% protein and 53% of a classical dermatan sulphate type glycosaminoglycan. The molecular weight of the proteoglycan was determined to be 130,000 and the molecular weight of the isolated glycosaminoglycan chains approximately 30,000. Thus the proteoglycan molecule should contain an average of two dermatan sulphate chains linked to a protein core of molecular weight approximately 61,000.

The skin proteoglycan gave a slightly lower buoyant density (1.42 gm/ml) on density gradient centrifugation. It appeared to be homogeneous on composite agarose polyacrylamide gel electrophoresis (relative mobility 0.71 - 0.73) and Sepharose 6-B gel chromatography (Kav 0.51) and contained 61% protein and 49% glycosaminoglycan. The glycosaminoglycans, probably of the dermatan sulphate type (containing only a small proportion of glucuronic acid residues), had a molecular weight of approximately 18,000. The molecular weight of the proteoglycan was determined to be 100,000 and hence the molecules should contain an average of two glycosaminoglycan chains linked to a protein core of approximately 61,000 molecular weight. Furthermore amino acid analysis of the two proteoglycan preparations showed that the respective cores had virtually identical composition (Table 18). Thus the skin and ligament proteoglycans appear to be essentially identical except for the chain length of their respective glycosaminoglycans.

Proteoglycans of somewhat similar composition have been isolated from bovine skin and heart valves (Toole and Lowther, 1968b), pig skin (Öbrink, 1972) and bovine tendon (Toole and Lowther, 1968b and Anderson, 1975). The proteoglycans extracted from heart valves with hot 6 M urea have been the most extensively characterized. They were shown to have a buoyant density of 1.55 gm/ml, a molecular weight between 100,000 and

200,000 (Preston, 1968), a protein content of approximately 50% and to contain 2 - 4 dermatan sulphate chains linked to a protein core of molecular weight 50,000 - 100,000. Dermatan sulphate proteoglycans isolated from the other tissues exhibited slight differences in protein and glycosaminoglycan content. It thus seems likely that this type of proteoglycan may be found associated with collagen (see later discussion) in all fibrous connective tissues. The present study further suggests that if the dermatan sulphate proteoglycans are isolated from various fibrous connective tissues and purified by the same techniques we may find that they have essentially identical protein cores and vary only in the substituent glycosaminoglycan chains.

Digestion of the dermatan sulphate proteoglycans isolated from the skin and periodontal ligament with cathepsin B and elastase gave rise to some polysaccharide material of similar mobility to the free glycosaminoglycan chains on composite agarose polyacrylamide gel electrophoresis. However, digestion with cathepsin D produced larger fragments indicative of the presence of multiple glycosaminoglycan chains linked to small peptides. If we propose that these proteoglycans contain only two glycosaminoglycan chains per molecule this would suggest that they both are linked to a small region of the protein core and that the major part of the protein is free of polysaccharide chains (Pearson et al, 1978b).

Incubation of the bovine skin and periodontal ligament dermatan sulphate proteoglycans at 37° C and pH 7.2 for 24 hrs. gave rise to a considerable proportion of smaller proteoglycan material that appeared to be breakdown products (Fig. 30). The relative mobility on composite agarose-polyacrylamide gel electrophoresis (skin 0.85, ligament 0.78) suggested the fragments were slightly larger than those produced by cathepsin D and thus contain a larger fragment of the protein core. Since this effect was only observed at close to neutral pH and was inhibited by the presence of 1.8 mM PMSF, it was attributed to the action of a serine protease present in these preparations. There was some evidence to suggest that the activity was inhibited in less pure preparations and became more active with proteoglycan purification.

If the degradation observed is, as we propose, due to the presence of a neutral protease, the enzyme must be either very resistant to denaturation or capable of efficient renaturation, since it was not destroyed by the strong denaturing agents (7 M urea and 4 M guanidinium chloride) used in the preparation of the proteoglycans. Furthermore it must be firmly attached to the proteoglycan even after density gradient centrifugation in 4 M guanidinium chloride.

Chance attachment of the protease and the proteoglycan could have occurred due to the conditions of extraction and purification of the proteoglycans. However if the enzyme was associated with the degradation of the proteoglycan in vivo we might have expected to find some of the smaller proteoglycan fragments in the extracts. The gel formed at the top of the gradient after density gradient centrifugation of the 0.1 M NaCl extracted periodontal ligament proteoglycans contained two sharp bands of proteoglycan material (Fig. 23). One had precisely the same mobility (R_{bpb} 0.78) as the degradation product obtained by incubation of the ligament dermatan sulphate proteoglycan at 37° C and similar to these degradation products, stained clearly with toluidine blue but was not detected with Coomassie blue. The other proteoglycan band had the mobility (R_{bpb} 0.66) and staining properties of the intact dermatan sulphate proteoglycan. Thus there is evidence to suggest that the enzyme is active on the proteoglycan at least before the tissue was extracted with guanidinium chloride and perhaps in vivo.

The possibility of a proteinase attached to the proteoglycan in vivo is, perhaps, not as outlandish as it might have been thought a few years ago. Immunocytochemical studies have shown that collagenase is found in association with collagen fibres in extracellular sites in most connective tissues (Dingle, 1976 and Montfort and Pérez-Tamayo, 1975). Furthermore the collagenase appears to be firmly bound to the collagen fibres since it has also been found in a number of purified collagen preparations (Pardo and Pérez-Tamayo, 1975). Dingle (1976) also had evidence to suggest that a protease may be bound to the proteoglycan matrix in cartilage explants in organ culture. He incorporated ^{35}S labelled proteoglycans (purified by conventional means) into polyacrylamide gels of a pore size that enabled the release of degradation products but not the undegraded proteoglycan. The gels were placed in close proximity to cartilage explants in organ culture and, even though the cartilage was rapidly degraded under stimulation with Vitamin A, no effect on the acrylamide proteoglycan gel could be demonstrated. Though a number of possible explanations were proposed, one was that protease in an inactive form was bound to the proteoglycan matrix in the cartilage but was not present in the proteoglycan gel because it had been removed on purification of the proteoglycan. The apparent protease attachment to the dermatan sulphate proteoglycans, however, must be much stronger than that proposed with cartilage explants.

I recognize that further experiments are required to establish the enzymic nature of the degradation observed with the dermatan sulphate proteoglycans, however the lability of these molecules is common to at least two different preparations from different connective tissues and may be characteristic of this type of proteoglycan. Furthermore accumulating evidence suggests that the attachment of protease, in an inactive form, to their substrates in the extracellular matrix may be a general phenomenon. The control of the turnover of the extracellular matrix may thus involve activation of the protease or removal of associated inhibitors.

Another characteristic of the skin and periodontal ligament proteoglycan revealed by incubation at 37° C. is their apparent ability to aggregate. In addition to the appearance of smaller apparently degraded proteoglycan products after incubation at 37° C, slower moving bands, representing apparently larger proteoglycan material, were observed on composite agarose-polyacrylamide gel electrophoresis. These are believed to arise from the hydrophobic interaction of proteoglycan molecules, since their relative proportions increased with warming and lower pH (Pearson et al, 1978b). Toole and Lowther (1968) and Öbrink (1972) also noticed that this type of proteoglycan had the ability to form larger aggregates and self-aggregation has recently been reported for cartilage proteoglycan (Sheehan et al, 1978) and for the keratan sulphate proteoglycan isolated from cornea (Axelsson and Heinegård, 1978).

Sheehan et al (1978) prepared an essentially monodisperse cartilage proteoglycan preparation by dissociative extraction and density gradient centrifugation (D-1-1 fraction of Hardingham et al, 1976) and found that molecular weights determined by light scattering varied with ionic strength; in buffered 150 mM NaCl, pH 7.4, the proteoglycan had a particle weight of about 5 x 10^6, but at 100, 200 and 300 mM NaCl particle weights of 2.5 x 10^6 - 3 x 10^6 were observed. These results, together with corroborating evidence from sedimentation velocity experiments, were interpreted in terms of proteoglycans self associating at physiological ionic strength. These authors suggested that lower ionic strength results in severe repulsions between the charged glycan chains on the proteoglycans, whereas higher ionic strength inhibited the binding at the protein site(s).

Axelsson and Heinegård (1978) found that the keratan sulphate proteoglycan monomers could form aggregates as determined by gel chromatography and sedimentation velocity analyses. The aggregation was promoted by low pH and reduction and alkylation of disulphide bonds and could not be attributed to the presence of any non-proteoglycan material. It is not known at present whether the self-aggregation of any of the proteoglycans occurs in vivo or is an artifact caused by the purification procedure.

Incubation of the skin and ligament dermatan sulphate proteoglycans in the presence of PMSF not only inactivated the apparent protease activity but also greatly enhanced the degree of aggregation. The nature of this effect is unknown, though the two phenomena may be linked. The action of the protease could cleave regions of the protein core involved in aggregation. However those proteoglycans that remain apparently unchanged after incubation at 37° C appear less able to aggregate than if incubated in the presence of PMSF. Only a fraction of the proteoglycan aggregated in the absence of PMSF, whereas only one band representing apparently aggregated proteoglycan was observed after incubation in the presence of PMSF. Thus another action of the PMSF may be to modify certain reactive groups or the conformation of the protein core such as to enhance aggregation.

Fig. 36 depicts a schematic model for the structure of the skin or periodontal ligament dermatan sulphate proteoglycan. The dermatan sulphate proteoglycans appear to be relatively small containing only approximately two dermatan sulphate chains per molecule and a protein

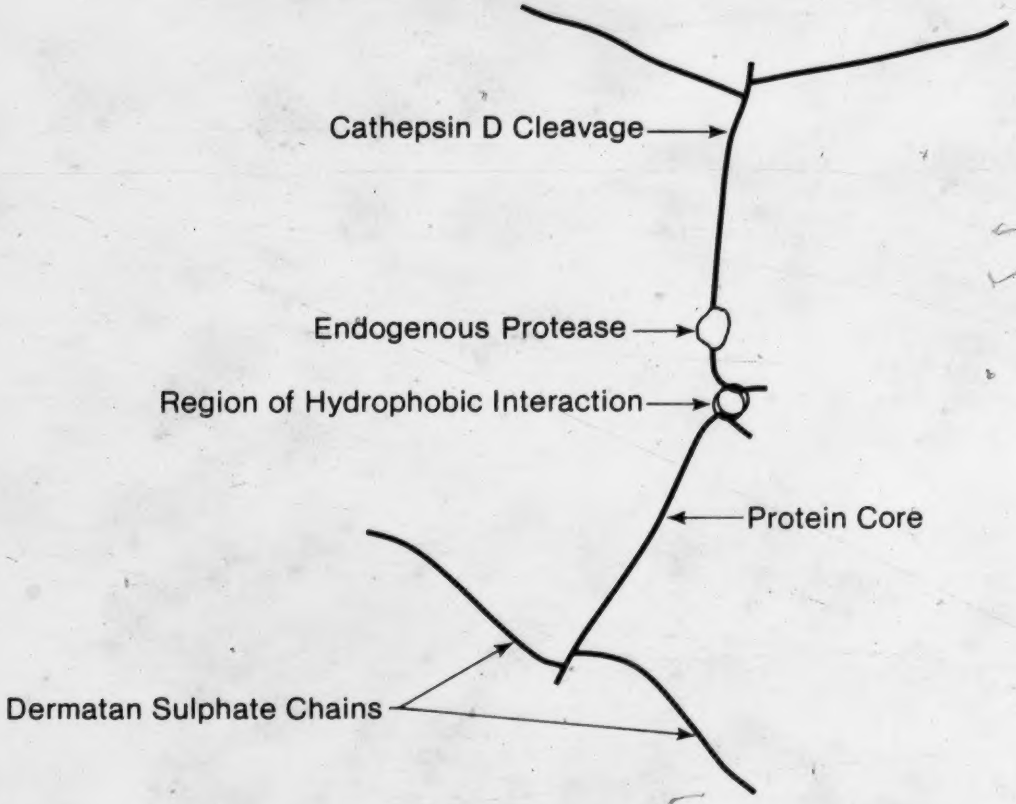

Cathepsin D Cleavage
Endogenous Protease
Region of Hydrophobic Interaction
Protein Core
Dermatan Sulphate Chains

Figure 36. Schematic Model of Dermatan Sulphate Proteoglycan

core of molecular weight approximately 61,000. They appear to contain a very closely associated neutral protease and have a proclivity to self-aggregation. Although extensive steps were taken to avoid degradation we cannot rule out the effects of protease activity or chemical modification during purification of the proteoglycans. However the homogeneity of the dermatan sulphate proteoglycans and very close similarity of the protein cores of the skin and periodontal ligament preparations suggest that if protelytic cleavage has occurred it must have been specific and limited.

There is strong evidence to suggest that the dermatan sulphate proteoglycans are closely associated with collagen in the fibrous connective tissues. The bulk of the dermatan sulphate proteoglycan can only be extracted from bovine periodontal ligament with 4 M guanidinium chloride; 0.4 M guanidinium chloride may extract a large proportion of the dermatan sulphate from bovine skin though this proteoglycan was not thoroughly characterized. Furthermore there is evidence to suggest that a large proportion of the dermatan sulphate proteoglycan extracted in 0.1 M NaCl may be a degradation product of the type formed by incubation of the dermatan sulphate proteoglycan at 37° C (see p. 191). Toole and Lowther (1965) also had evidence to suggest that the dermatan sulphate proteoglycans extractable in 1.0 M NaCl were degradation products. These preparations were found to have considerably lower protein contents and sedimentation coefficients than the proteoglycans extractable in hot urea (proteoglycans similar to the skin and ligament proteoglycans reported in this thesis).

The extraction, using low salt solutions, of dermatan sulphate proteoglycans that appear to contain the glycosaminoglycan region of the native proteoglycan but have a degraded protein core suggests that the protein core is involved in binding to the collagen fibres. Toole (1976) also found that the binding of cartilage proteoglycan and precipitation of collagen was enhanced in the presence of PMSF and attributed this to the inactivation of endogenous protease present in the proteoglycan preparations (isolated from chick limb bud or embryo sternae with 4 M guanidinium chloride and purified by CTAB and alcohol precipitation). These findings are consistent with the observation that isolated core protein, from cartilage proteoglycan, bound to collagen fibres covalently linked to an affinity column (Greenwald et al, 1975) or to collagen during fibril formation in vitro (Oegema et al, 1975b), whereas the free chondroitin sulphate chains had no affinity for the collagen at physiological ion strength in these experiments. However, competition for binding between core protein and intact proteoglycan could not be demonstrated (Greenwald et al, 1975, Oegema et al, 1975b and Toole, 1976). The explanation for these apparently contradictory results might lie in the complex nature of the proteoglycan collagen interaction. It appears likely that binding may occur between collagen and either the chondroitin sulphate chains (Öbrink, 1973, Greenwald et al, 1975 and Öbrink and Sundelöf, 1973), the core protein (Greenwald et al, 1975 and Oegema et al, 1975b) or the intact proteoglycan (Oegema et al, 1975b, Öbrink and Sundelöf, 1973, Greenwald et al, 1975 and Toole, 1976), but that the binding of the core protein is considerably stronger than that of chondroitin sulphate chains. Thus it is likely that the primary specific binding site lies in the core protein, but that this reaction gives rise to a spatial arrangement which maximizes secondary interactions between chondroitin sulphate chains and basic residues in the collagen molecules.

It has been suggested that proteoglycans also affect the organization of collagen fibres. Loewi and Meyer (1958) have shown that dermatan sulphate is found in those tissues where there are coarse or thick collagen fibres, but not in tissues containing much finer fibres such as cornea or cartilage. Borcherding et al (1975) correlated the number and size of collagen fibres with the concentration and composition of glycosaminoglycans at discrete intervals across the transition zone between the cornea and sclera. They found there was a rapid increase in fibre size and decrease in organization accompanied by a rapid decrease in keratan sulphate and increase in dermatan sulphate concentration. Considering the familial similarity of the protein cores of the dermatan sulphate proteoglycan and the keratan sulphate proteoglycan (Table 18), it is interesting to speculate that these two proteoglycans may compete for the primary binding site on the collagen molecule and whereas the keratan sulphate chains discourage the formation of thicker fibres (the free chains do not appear to bind to collagen, Öbrink, 1973), the dermatan sulphate chains interact strongly at secondary binding sites on the collagen molecules to encourage the formation of thicker collagen fibres, perhaps by linking fibres, as observed under the electron microscope (Myers et al, 1973) or by a similar mechanism stabilizing the formation of larger collagen bundles.

There is good evidence that the interaction of glycosaminoglycan chains with collagen is dependant on the chain length of the glycosaminoglycans (Öbrink and Wasteson, 1971). Öbrink and Sundelöf (1973) found that whereas 2 to 4 dermatan sulphate molecules of 18,000 molecular weight could bind to each collagen molecule, a single high molecular weight (41,000) dermatan sulphate of similar iduronic acid composition could bind as many as 5 molecules of collagen per chain. This may suggest that the periodontal ligament dermatan sulphate proteoglycan may give rise to thicker collagen fibres than the skin dermatan sulphate proteoglycan since the only apparent difference in the proteoglycans is their polysaccharide chain length (33,000 c.f. 18,000 molecular weight). Unfortunately the precise fibre diameter of collagen fibres from the periodontal ligament has not been reported, though thick bundles of collagen fibres (the principal fibres) are known to occur. The proposed difference in dermatan sulphate proteoglycan-collagen binding may also explain why newly synthesized collagen in the periodontal ligament was quantitatively converted to insoluble collagen, whereas in the skin a conversion efficiency of only 33% was found (Sodek, 1977).

6.3 CHANGES IN THE LIGAMENT GLYCOSAMINOGLYCANS ASSOCIATED WITH TOOTH DEVELOPMENT

The growth and development of the permanent incisor was accompanied by marked changes in the structural organization of the periodontal ligament. Histological examination revealed an obvious increase in collagen fibre organization and orientation with the first signs of root formation (group 2 incisors). The fibres were much thicker than those of the follicle and organized in wavy bundles, parallel to the root surface (Plate 1). In support of these observations, chemical analyses revealed an almost two fold increase in the collagen content and also a dramatic decrease in the hyaluronic acid content of the ligament at this stage of development.

The hyaluronic acid analyses obtained are consistent with the work of Pearson et al (1975), who showed that hyaluronic acid decreased relative to chondroitin sulphate in the periodontal ligament with the development of bovine molars and with the general observation that hyaluronic acid is more concentrated in embryonic mesenchymal tissues than in their older counterparts (Loewi and Meyer, 1958 and Breen et al, 1970). Furthermore hyaluronate synthesis has been shown to be associated with the early stages of growth and reparative processes involving new synthesis of connective tissue elements and the active migration and proliferation of the participating cells in, for example, wound healing (Bentley,1967), tendon regeneration (Dorner, 1968), newt limb regeneration (Toole and Gross, 1971), chick embryo cornea development (Toole and Trelstad, 1971), limb bud and axial chondrogenesis (Toole, 1972) and the fetal development of human skin (Breen et al, 1970). Small amounts of hyaluronic acid have been shown to inhibit the synthesis of chondrocyte specific glycosaminoglycans by chondrocytes in tissue culture (Weibkin and Muir, 1973) and removal of the hyaluronic acid, presumably by a hyaluronidase, has been proposed to cause the immobilization of chondrocytes and allow their differentiation during limb bud chondrogenesis (Toole, 1973). However hyaluronic acid does not appear to effect the synthesis of sulphated glycosaminoglycans by dermal fibroblast in tissue culture (Weibkin and Muir, 1973) suggesting that the effect of hyaluronic acid on cell differentiations varies with different cell types. However the presence of large amounts of hyaluronic acid in the follicle prior to root and ligament formation is consistent with the embryonic nature of this tissue and the organization and development of presumptive cementoblasts and ligament fibroblasts.

Subsequent development of the periodontal ligament revealed little obvious histological change until the incisor erupted into the oral cavity. Obliquely oriented collagen fibre bundles were first observed in the newly erupted incisors and became the predominant fibre-type in the mature occluded incisors. The proposed function of the periodontal ligament in pulling the tooth into occlusion by the action of contractile fibroblasts attached to the collagen fibres, is compatible with an oblique orientation of collagen fibres that are firmly attached to the cement (Beertsen *et al*, 1974). Failure to observe these fibres in the earlier stages of incisor development casts some doubt on the involvement of the periodontal ligament in this process prior to eruption into the oral cavity. It is possible that fibre orientation was lost in removing the incisors from the fractured mandibles, however fibre orientation in the cement of the mature occluded incisors suggested that the oblique orientation of collagen fibres occurred late in the development of the cement (Plate 3,e) and the histological observation of developing monkey premolars, sectioned while still within the mandible, suggested a very similar sequence of ligament development (Grant and Bernick, 1972).

Although it was not always obvious from histological examination, hydroxyproline analysis revealed a steady increase in the collagen content of the ligament with development. This probably reflects an increasing requirement for mechanical strength which may be expected to reach a maximum in the ligament of the mature occluded incisors.

While little change was observed in the hyaluronic acid content of the ligament at these stages of development a marked change in the galactosaminoglycans was observed. Isolation of the alcohol fractionated galactosaminoglycans showed that though there appeared to be little change in the composition of the individual fractions there were marked changes in the amounts present. The 18 and 25% alcohol fractions showed approximately parallel changes with development as did the 40 and 50% alcohol fractions, consistent with the proposal that most of the dermatan sulphate-like galactosaminoglycans, that is the 18 and 25% alcohol fractions, and the chondroitin sulphate-like galactosaminoglycans, that is the 40 and 50% alcohol fractions, are present in distinctly different proteoglycans.

The dermatan sulphate-like glycosaminoglycans increased steadily with development and approximated the changes observed in the ligament collagen content. Similar changes in dermatan sulphate content have been observed with the deposition of collagen fibres and the development of human skin (Breen *et al*, 1970), pig skin (Loewi and Meyer, 1958) and chick skin (Kawamoto and Nagai, 1976). As previously mentioned, dermatan sulphate and dermatan sulphate proteoglycan have long been implicated in the formation and organization of collagen fibres. Thus the presence of dermatan sulphate in the periodontal ligament is compatible with the fibrous nature of this tissue and it is not surprising that changes in the amounts present closely reflect changes in collagen content.

The chondroitin sulphate-like glycosaminoglycans increase rapidly with development to reach a maximum content in the newly erupted incisors, then decrease slightly in the mature fully occluded incisor. From the analysis of the chondroitin sulphate-type proteoglycan from the ligament it seems likely that this proteoglycan is similar to the cartilage type proteoglycans. As such it would be well suited, because of its greater water-inclusion properties, to protect both the cells and collagen from increased pressure as the ligament is compressed between the alveolar bone and the cement. Dramatic alteration in the proteoglycan content in relation to compression or tension of the extracellular matrix has been shown in the rabbit flexor digitorum profundus tendon (Gillard et al, 1977). This tendon is subjected to either compressive or tensile forces depending on its position in the leg or foot of the rabbit. Where the tendon was under tension the tissue contained less than 0.2% proteoglycans, on a dry weight basis, composed predominantly of dermatan sulphate. In the area of compression, however, the tendon contained approximately 3.5% proteoglycan composed of predominantly chondroitin sulphate. Accompanying the change from predominantly dermatan sulphate to predominantly chondroitin sulphate glycosaminoglycans, the authors observed a decrease in the axial periodicity of the collagen fibres from 63 to 54 nm. From these and studies of the staining properties of collagen fibres, under tension and relaxation (Flint et al, 1975), they proposed that changes in the distribution of freely available fixed charges, which are reflected in differences in the axial periodicity and staining of collagen fibres, could be responsible for changes in the cellular metabolic activity, which gives rise to the modulation in proteoglycan composition of the extracellular matrix.

However, although the amount of compressive force produced within the periodontal ligament and the changes in this force with development are unknown, it might be expected to reach a maximum in mature teeth due to the forces involved in occlusion. The fact that the chondroitin sulphate-like glycosaminoglycans reach a maximum in the incisor periodontal ligament prior to occlusion suggests that the associated proteoglycans may have additional functions in this tissue.

Studies of embryonic salivary glands have shown that branching morphogenesis is dependant on the presence at the epithelial surface of a proteoglycan complex, composed of approximately equal amounts of hyaluronic acid and chondroitin sulphate (Cohn et al, 1977). The accumulation of newly synthesized proteoglycan is greatest in areas of incipient cleft formation (Bernfield et al, 1972) and clefts are lost after removal of surface proteoglycans and reappear coincident with the accumulation of newly synthesized proteoglycan at specific sites (Banerjee et al, 1977). Contractility of microfilaments within the cells of the epithelium has been proposed to cause the cells to change in shape and to initiate the formation of clefts (Spooner and Wessells, 1970). The contractility of microfilaments is dependent on the availability of Ca^{2+} (Wessells et al, 1971) and Bernfield et al, (1972) proposed that surface associated proteoglycans because of their high affinity for Ca^{2+} may be involved in regulating Ca^{2+} access to the plasma membrane and/or the cell interior and thus involved in promoting cleft formation.

The eruption of teeth has also been proposed to involve the contractile mechanism of microfilaments, in this case within the fibroblast (Beertsen, 1975). A similar involvement of the chondroitin sulphate proteoglycans with the process of contraction may also be envisaged in this tissue. The greatest rate of eruption may be expected just prior to occlusion, since the resisting forces are lowest in this situation (Berkovitz and Thomas, 1969). Thus a high rate of eruption may account for the high levels of chondroitin sulphate-type glycosaminoglycans found in the periodontal ligament just prior to occlusion of the bovine incisor.

The periodontal ligament contains two predominant types of galactosaminoglycans and probably two corresponding types of proteoglycan, both of which may play vital roles in the development, organization and normal functioning of the periodontal ligament. The dermatan sulphate proteoglycan is probably involved in the formation and organization of collagen fibres and the chondroitin sulphate proteoglycans with the resistance to compressive forces and possibly also with the contraction of microfilaments within the fibroblasts. Proteoglycans may have some association with oxytalin (known to be present in considerable quantities in bovine periodontal ligament), however very little is known concerning oxytalin- or elastin-proteoglycan interaction.

The presence of two distinct types of proteoglycan within the periodontal ligament suggests that changes in the relative proportions of the dermatan sulphate type and chondroitin sulphate type glycosaminoglycans involve a regulation of the synthesis of distinct proteoglycans rather than a modulation at the level of glycosaminoglycan synthesis, such as in epimerase or sulphotransferase activity. However modulation of epimerase or sulphotransferase activity may give rise to some modification of the copolymeric nature of the glycosaminoglycans of the individual proteoglycans. This may amount to changes in a relatively small proportion of the disaccharide units of the glycosaminoglycans and could produce a variation such as is observed between the 18 and 25% alcohol fractions.

6.4 SUGGESTIONS FOR FURTHER WORK

As well as giving some insight into the nature of the periodontal ligament proteoglycans and non cartilaginous proteoglycans in general, it is hoped that the work reported in this thesis will stimulate further research; not only to confirm some of the findings already suggested but also to extend these investigations to further examine the nature of the chondroitin sulphate and dermatan sulphate proteoglycans. One area of investigation that is currently receiving a great deal of interest is the nature of the non cartilaginous chondroitin sulphate proteoglycans and the comparison of the characteristics of these molecules with those of the proteoglycans isolated from cartilage.

The characteristics of the apparent protease associated with the dermatan sulphate proteoglycans also warrants further investigation.

We hope to make extensive efforts to isolate the apparent protease activity from the proteoglycan. Gel chromatography in SDS or urea may be effective in this regard. Isolation of the enzyme free from the proteoglycan would facilitate more extensive characterization and should indicate whether this enzyme corresponds to any of the proteases already isolated from connective tissues (Barrett, 1975 and Dingle, 1976).

Further investigation of the apparent aggregation phenomenon of the dermatan sulphate proteoglycans is planned for the near future. We have already shown that the apparent aggregation phenomenon can be demonstrated on gel chromatography in the presence of 0.5 M sodium acetate (Pearson et al, 1978b). These results suggest that the association is unlikely to be an ionic interaction (for example between a positively charged region of the core protein and the glycosaminoglycan chains of another proteoglycan molecule). The results also suggest that the apparent aggregation is not simply a masking of negative charges, which was a possible interpretation of the decreased migration toward the positive pole observed in gel electrophoresis.

If the apparent increase in proteoglycan size on incubation at 37° C is a simple aggregation phenomenon it should be easily reversible. We propose to investigate this by incubation of highly purified dermatan sulphate proteoglycan preparations at 37° C in the manner described previously (section 2.2.8) and isolation of any apparently aggregated material by gel chromatography. Initial work, performed in Dr. Pearson's laboratory, has shown that the apparent aggregation phenomenon, observed on composite agarose polyacrylamide gel electrophoresis, is inhibited by incubation in the presence of 6 M urea, suggesting that urea may be effective in dissociating the aggregated proteoglycan. We thus plan to treat the "aggregates" with 6 M urea and isolate the products by gel chromatography. Isolation of proteoglycans the size of the "subunit" material should facilitate the demonstration of subsequent reaggregation after incubation at 37° C. Reversible changes demonstrated in this manner would suggest the occurrence of an aggregation phenomenon, however reversible conformational changes would not be eliminated.

Analytical ultracentrifugation may also be used to demonstrate the aggregation of the dermatan sulphate proteoglycan. We have shown that the bovine skin and periodontal ligament dermatan sulphate proteoglycans give single sharp peaks on sedimentation velocity analysis at 20° C (unpublished results). If aggregation occurs a faster moving peak would be expected after incubation at 37° C. These studies or the use of equilibrium sedimentation analysis should eliminate effects due to extensive conformational changes and also give an indication of the extent of aggregation. Some caution should, however, be exercised in the interpretation of a negative result. The failure to observe any larger molecular weight material in the ultracentrifuge may be due to the instability of aggregates under the forces produced during ultracentrifugation. Such an instability is observed with the aggregates of hyaluronic acid and cartilage proteoglycan in the absence of link proteins (Muir and Hardingham, 1975).

Assuming that aggregation is proved the nature of the association, whether ionic or hydrophobic, and the site(s) on the dermatan sulphate proteoglycan involved in aggregation could readily be established by modification (either enzymic or chemical) of the proteoglycan structure; the stability and possible extent of aggregation could be revealed by varying the conditions under which the aggregates are formed.

An area of investigation touched on in this thesis, which I feel may prove very rewarding in the future, is the investigation of the binding of dermatan sulphate proteoglycans to collagen fibres and isolated collagen molecules. Though collagen-proteoglycan interaction has been investigated for many years, the availability of two relatively simple, well characterized dermatan sulphate proteoglycans may open a number of opportunities for a fresh approach to this problem. Limited enzymic cleavage (cleavage of the glycosaminoglycan chains and/or partial proteolytic cleavage of the protein core) combined with collagen binding studies (e.g. collagen affinity chromatography) should give some idea of the location of the collagen binding sites on the dermatan sulphate proteoglycan. These studies should reveal the relative involvement of the glycosaminoglycan chains and the protein core in binding to collagen and hopefully give a more detailed understanding of the structure of the collagen binding region of the protein core if, in fact, such a region exists. By use of competitive binding studies it may be possible to show whether these proteoglycans (and possibly isolated binding regions) compete with other proteoglycans (e.g. keratan sulphate proteoglycans and chondroitin sulphate proteoglycans) for the same binding sites or whether they bind to distinct sites on the collagen molecule. Comparative studies of collagen-proteoglycan interaction, using the skin dermatan sulphate proteoglycan and the periodontal ligament dermatan sulphate proteoglycan, may give some indication of the effect the chain length of the constituent glycosaminoglycans has on collagen fibre formation, fibre stability and perhaps fibre form.

BIBLIOGRAPHY

Anderson, B., Hoffman, P. & Meyer, K. (1965) J. Biol. Chem. 240, 156-167

Anderson, D.J., Hannam, A.G., & Mathews, B. (1970) Physiol. Rev. 50, 171-195

Anderson, J.C. (1975) Biochim.Biophys. Acta 379, 444-455

Antonopoulos, C.A., Axelsson, I., Heinegård, D. & Gardell, S. (1974), Biochim. Biophys. Acta 338, 108-119

Arnold, E.A., Yawn, D.H., Brown, D., Wyllie, R.C. & Coffey, D.S. (1972) J. Cell Biol. 53, 737-757

Axelsson, I. & Heinegård, D. (1975) Biochem. J. 145, 491-500

Axelsson, I. & Heinegård, D. (1978) Biochem. J. 169, 517-530

Bailey, A.J. & Robins, S.P. (1976) Sci. Prog., Oxf. 63, 419-444

Baker, J. & Caterson, B. (1977) Biochem. Biophys. Res. Commun. 77, 1-10

Baker, J.R., Cifonelli, J.A. & Rodén, L. (1975) Connect. Tissue Res. 3, 149-156

Banerjee, S.D., Cohn, R.H. & Bernfield, M.R. (1977) J. Cell Biol. 73, 445-463

Barrett, A.J. (1975) in Proteases and Biological Control (Reich, E., Rifkin, D. & Shaw, E., eds.), pp. 467-482, Cold Spring Harbor Laboratory, New York

Baumhammers, A. & Stallard, R.E. (1968) J. Periodont. Res. 3, 187-193

Beertsen, W. (1975) Arch. Oral Biol. 20, 659-666

Beertsen, W. & Everts, V. (1977) Anat. Rec. 189, 479-498

Beertsen, W., Everts, V. & Van der Hooff, A. (1974) Arch. Oral Biol. 19, 1087-1098

Bently, J.P. (1967) Ann. Surg. 165, 186-191

Bergqvist, U., Samuelsson, G. & Uvnäs, B. (1971) Acta Physiol. Scand. 83, 362-372

Berkovitz, R.K.B. (1971) Arch. Oral Biol. 16, 1033-1043

Berkovitz, R.K.B. & Thomas, N.R. (1969) Arch. Oral Biol. 14, 771-780

Bernfield, M.R., Banerjee, S.D. & Cohn, R.H. (1972) J. Cell Biol. 52, 664-673

Bettelheim, F.A. (1970) in Biological Polyelectrolytes (Veis, E., ed.), pp. 131-209, M. Dekker, New York

Bhavanandan, V.P. & Davidson, E.A. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 2032-2036

Bien, S.M. (1966) Adv. Oral Biol. 2, 173-201

Bitter, T. & Muir, H. (1962) Anal. Biochem. 4, 330-334

Blaschke, E., Bergqvist, U. & Uvnäs, B. (1976) Acta Physiol. Scand. 97, 110-120

Borcherding, M.S., Blacik, L.J., Sittig, R.A., Bizzell, J.W., Breen, M. & Weinstein, H.G. (1975) Exp. Eye Res. 21, 59-70

Bray, B.A., Lieberman, R. & Meyer, K. (1967) J. Biol. Chem. 242, 3373-3380

Breen, M., Weinstein, H.G., Johnson, R.L., Veis, A. & Marshall, R.T. (1970) Biochim. Biophys. Acta 201, 54-60

Brunette, D.M., Melcher, A.H. & Moe, H.K. (1976) Arch. Oral Biol. 21, 393-400

Buddecke, E. & Schubert, M. (1961) Hoppe-Seyler's Z. Physiol. Chem. 325, 189-203

Butler, W.T., Birkedal-Hansen, H., Beegle, W.F., Taylor, R.E. & Chung, E. (1975) J. Biol. Chem. 250, 8907-8912

Carmichael, G.G. (1968) Arch. Oral Biol. 13, 765-772

Castellino, F.J., Brockway, W.J., Thomas, J.K., Lino, H.T. & Rawitch, A.B. (1973) Biochemistry 12, 2787-2791

Caterson, B. & Baker, J. (1978) Biochem. Biophys. Res. Commun. 80, 496-503

Chain, E. & Duthie, E.S. (1940) Brit. J. Exp. Path. 21, 324-338

Chervenka, C.H. (1969) A Manual of Methods for the Analytical Ultracentrifuge, Beckman Instruments, Palo Alto

Chung, E. & Miller, E.J. (1974) Science 183, 1200-1201

Cohn, R.C., Banerjee, S.D. & Bernfield, M.R. (1977) J. Cell Biol. 73, 464-478

Comper, W.D. & Laurent, T.C. (1978) Physiol. Rev. 58, 255-315

Conochie, L.B., Scott, J.E. & Page Faulk, W. (1975) J. Immunol. Methods 7, 393-398

Constantopoulos, G., Dekaban, A.S. & Carroll, W.R. (1969) Anal. Biochem. 31, 59-70

Cook, R.T. & Aikawa, M. (1973) Exp. Cell Res. 78, 257-270

Cöster, L., Malmström, A., Sjöberg, I. & Fransson L.-Å. (1975) Biochem. J. 145, 379-389

Cuev[illegible]ro, L.A., Pita, J.C. & Howell, D.S. (1973) Calcif. Tissue Res. 13, 1-10

Cunningham, W.M. (1971) N.Z. Dent. J. 67, 5-12

Di Ferrante, N., Donnelly, P.V. & Berglund, R.K. (1971) Biochem. J. 124, 549-553

Dingle, J.T. (1976) in Proteolysis and Physiological Regulation (Ribbons, D.W. & Brew, K., eds.) pp. 339-355, Academic Press, New York

Dische, Z. (1947) J. Biol. Chem. 167, 189-198

Dorner, R.W. (1968) Arch. Biochem. Biophys. 128, 34-39

Eastae, J.E. & Melcher, A.H. (1971) J. Dent. Res. 50, 675

Ehrlich, K.C., Radhakrishnamurthy, B. & Berenson, G.S. (1975) Arch. Biochem. Biophys. 171, 361-369

Einarsson, R. & Andersson, L-O. (1977) Biochim. Biophys. Acta 490, 104-111

Eisenstein, R., Larsson, S.-E., Kuettner, K.E., Sorgente, N. & Hascall, V.C. (1975) Atherosclerosis 22, 1-17

Epstein, E.H. (1974) J. Biol. Chem. 249, 3225-3231

Eyre, D.R. & Muir, H. (1974) FEBS Lett. 42, 192-196

Eyring, E.J. & Yang, J.T. (1968) Biopolymers 6, 694-701

Farber, S.J., Schubert, M. & Schuster, N. (1957) J. Clin. Invest. 36, 1715-1722

Flint, M.H., Lyons, M.F., Meaney, M.F. & Williams, D.E. (1975) Histochem. J. 7, 529-546

Flory, P.J. (1953) Principles of Polymer Chemistry, p.317, Cornell University Press, New York

Franek, M.D. & Dunstone, J.R. (1966) Biochim.Biophys. Acta 127, 213-222

Fransson, L.-Å. (1968) J. Biol. Chem. 243, 1504-1510

Fransson, L.-Å. (1974) Carbohyd. Res. 36, 339-348

Fransson, L.-Å. (1976) Biochim. Biophys. Acta 437, 106-115

Fransson, L.-Å., Antonopoulos, C.A. & Gardell, S. (1970) Carbohyd. Res. 15, 73-89

Fransson, L.-Å. & Carlstedt, I. (1974) Carbohyd. Res. 36, 349-358

Fransson, L.-Å., Cöster, L., Malmström, A. & Sjöberg, I. (1974) Biochem. J. 143, 369-378

Fransson, L.-Å. & Havsmark, B. (1970) J. Biol. Chem. 245, 4770-4783

Fransson, L.-Å. & Malmström, A. (1971) Eur. J. Biochem. 422-430

Fransson, L.-Å., Malmström, A., Lindahl, U. & Hook, M. (1973) in Biology of the Fibroblast (Kulonen, E. & Pikkarainen, J., eds.), pp.440-448, Academic Press, New York

Fransson, L.-Å. & Rodén, L. (1967a) J. Biol. Chem. 242, 4161-4169

Fransson, L.-Å. & Rodén, L. (1967b) J. Biol. Chem. 242, 4170-4175

Freedman, H.L. & Grainger, R.M. (1965) J. Can. Dent. Assoc. 31, 779-786

Freeman, E., Ten Cate, A.R. & Dickinson, J. (1975) Arch. Oral Biol. 20, 139-140

Fromme, H.G., Buddecke, E., Von Figura, K. & Kresse, H. (1976) Exp. Cell Res. 102, 445-449

Fullmer, H.M. (1967) in Structural and Chemical Organization of Teeth (Miles, A.E.W., ed.), pp. 349-414, Academic Press, New York

Gabbiani, G., Hirschel, B.J., Ryan, G.B., Statkov, P.R. & Manjo, G. (1972) J. Exp. Med. 135, 719-734

Gabel, A.B. (1956) J. Periodont. 27, 191-198

Gelman, R.A. & Blackwell, J. (1974) Biochim. Biophys. Acta 342, 254-261

Gerding, J.J.T., Koppers, A., Hagel, P. & Bloemendal, H. (1971) Biochim. Biophys. Acta 243, 375-379

Giannattasio, G. & Zanini, A. (1976) Biochim. Biophys. Acta 439, 349-357

Gillard, G.C., Merrilees, M.J., Bell-Booth, P.G., Reilly, H.C. & Flint, M.H. (1977) Biochem. J. 163, 145-151

Goldstein, J., Waldman, A.A., & Marx, G. (1975) Adv. Exp. Med. Biol. 52, 289-297

Grant, D. & Bernick, S. (1972) J. Periodont. 43, 17-24

Greenwald, R.A., Schwartz, C.E. & Cantor, J.O. (1975) Biochem. J. 145, 601-605

Gross, J. & Kirk, D. (1958) J. Biol. Chem. 233, 355-360

Guis, M.E., Slootweg, R.N. & Tonino, G.J.M. (1973) Arch. Oral Biol. 18, 253-263

Habuchi, H., Yamagata, T., Iwata, H. & Suzuki, S. (1973) J. Biol. Chem. 248, 6019-6028

Hagel, P., Gerding, J.J.T., Fieggen, W. & Bloemendal, H. (1971) Biochim. Biophys. Acta 243, 336-373

Handin, R.I. & Cohen, H.J. (1976) J. Biol. Chem. 251, 4273-4282

Hardingham, T.E., Ewins, R.J.F. & Muir, H. (1976) Biochem. J. 157, 127-143

Hardingham, T.E. & Muir, H. (1972) Biochim. Biophys. Acta 279, 401-405

Hardingham, T.E. & Muir, H. (1974) Biochem. J. 139, 565-581

Hascall, V.C. (1977) J. Supramolecular Structure 7, 101-120

Hascall, V.C. & Heinegård, D. (1974a) J. Biol. Chem. 249, 4232-4241

Hascall, V.C. & Heinegård, D. (1974b) J. Biol. Chem. 249, 4242-4249

Hascall, V.C. & Riolo, R.C. (1972) J. Biol. Chem. 247, 4529-4538

Hascall, V.C. & Sajdera, S.W. (1969) J. Biol. Chem. 244, 2384-2396

Hascall, V.C. & Sajdera, S.W. (1970) J. Biol. Chem. 245, 4920-4930

Heinegård, D. (1973) Chem. Scr. 4, 199-201

Heinegård, D. (1977) J. Biol. Chem. 252, 1980-1989

Heinegård, D. & Axelsson, I. (1977) J. Biol. Chem. 252, 1971-1979

Heinegård, D. & Hascall, V.C. (1974) J. Biol. Chem. 249, 4250-4256

Helting, T. & Rodén, L. (1968) Biochim. Biophys. Acta 170, 301-308

Höök, M., Lindahl, U., Hallén, A. & Backstrom, G. (1975) J. Biol. Chem. 250, 6065-6071

Hopwood, J.J. & Robinson, H.C. (1973) Biochem. J. 135, 631-637

Hopwood, J.J. & Robinson, H.C. (1974) Biochem. J. 141, 517-526

Hopwood, J., Höök, M., Linker, A. & Lindahl, U. (1976) FEBS Lett. 69, 51-54

Howell, D.S. & Pita, J.C. (1976) Clin. Orthop. 118, 208-229

Inoue, S. & Iwasaki, M. (1976) J. Biochem. 80, 513-524

Inoue, S. & Miyawaki, M. (1975) Anal. Biochem. 65, 175-186

Iverius, P.-H. (1972) J. Biol. Chem. 247, 2607-2613

Kawamoto, T. & Nagai, Y. (1976) Biochim. Biophys. Acta 437, 190-199

Kefalides, N.A. (1975) J. Invest. Dermatol. 65, 85-92

Kinoshita, S. (1974) Exp. Cell Res. 85, 31-40

Körber, K.H. (1970) J. Periodont. 41, 382-390

Kresse, E., Heidel, H. & Buddecke, E. (1971) Eur. J. Biochem. 22, 557-562

Lagunoff, D., Phillips, M.T., Iseri, O.A. & Benditt, E.P. (1964) Lab. Invest. 13, 1331-1344

Lagunoff, D. & Warren, G. (1962) Arch. Biochem. Biophys. 99, 396-400

Laurent, T.C. & Killander, J. (1964) J. Chromatogr. 14, 317-330

Lear, C.S.C., Mackay, J.S. & Lowe, A.A. (1972) J. Dent. Res. 51, 1478-1482

Li, E.H.H., Fenton, J.W. & Feinman, R.D. (1976) Arch. Biochem. Biophys. 175, 153-159

Lillie, R.D. (1965) Histopathologic Technique and Practical Histochemistry, p.270, McGraw-Hill, New York

Lindahl, U. (1966) Biochim. Biophys. Acta 130, 368-382

Lindahl, U. (1976) in MTP Intern. Rev. Sci. Organic Chemistry Series Two, Carbohydrates (Aspinall, G.O., ed.), vol.7, pp. 283-312, Butterworth's, London

Lindahl, U. & Höök, M. (1978) Annu. Rev. Biochem. 47, 385-417

Lindahl, U., Höök, M., Backström, G., Jacobsson, I., Riesenfeld, J., Malmström, A., Rodén, L. & Feingold, D.S. (1977) Fed. Proc. 36, 19-23

Lindahl, U. & Rodén, L. (1966) J. Biol. Chem. 241, 2113-2119

Lloyd, K.O., Kabat, E.A., Layug, E.J. & Gruezo, F. (1966) Biochemistry 5, 1489-1501

Loewi, G. & Meyer, K. (1958) Biochim. Biophys. Acta 27, 453-456

Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. (1951) J. Biol. Chem. 193, 265-275

Lowther, D.A., Preston, B.N. & Meyer, F.A. (1970) Biochem. J. 118, 595-601

Lowther, D.A., Toole, B.P. & Meyer, F.A. (1967) Arch. Biochem. Biophys. 118, 1-11

Ludowieg, J., Vennesland, B. & Dorfman, A. (1961) J. Biol. Chem. 236, 333-339

Luscombe, M. & Phelps, C.F. (1967) Biochem. J. 103, 103-109

MacGregor, E.A. & Bowness, J.M. (1971) Can. J. Biochem. 49, 417-425

Majno, G., Gabbiani, G., Hirschel, B.J. Ryan, G.B. & Statkov, P.R. (1971) Science 173, 548-550

Malmström, A., Carlstedt, I., Aberg, L. & Fransson, L.-Å. (1975) Biochem. J. 151, 477-489

Malmström, A. & Fransson, L.-Å. (1971) Eur. J. Biochem. 18, 431-435

Malmström, A., Fransson, L.-Å., Höök, M. & Lindahl, U. (1975) J. Biol. Chem. 250, 3419-3425

Margolis, R.U. & Margolis, R.K. (1973) Biochem. Pharmacol. 22, 2195-2197

Margolis, R.U. & Margolis, R.K. (1977) Int. J. Biochem. 8, 85-91

Mashouf, K. & Engel, M.B. (1975) Arch. Oral Biol. 20, 161-166

Mathews, M.B. (1953) Arch. Biochem. Biophys. 43, 181-193

Mathews, M.B. (1956) Arch. Biochem. Biophys. 61, 367-377

Mathews, M.B. (1959) Biochim. Biophys. Acta 35, 9-17

Mathews, M.B. (1965) Biochem. J. 96, 710-716

Mathews, M.B. (1975) in Connective Tissue. Macromolecular Structure and Evolution (Mathews, M.B., Ed.), Springer-Verlag, New York

Mathews, M.B. & Lozaityte, I. (1958) Arch. Biochem. Biophys. 74, 158-174

Mayes, R.W., Mason, R.M. & Griffin, D.C. (1973) Biochem.J. 131, 541-553

Melcher, A.H. & Walker, T.W. (1976) in The Eruption and Occlusion of Teeth (Poole, D.F.G. & Stack, M.V., eds.), pp.183-192, Butterworth's, London

Meyer, F.A., Preston, B.N. & Lowther, D.A. (1969) Biochem. J. 113, 559-563

Meyer, K. (1966) Fed. Proc. 25, 1032-1034

Meyer, K., Davidson, E., Linker, A. & Hoffman, P. (1956) Biochim. Biophys. Acta 21, 506-518

Meyer, K., Linker, A., Davidson, E.A. & Weissman, G. (1953) J. Biol. Chem. 205, 611-616

Miller, E.J. (1976) Mol. & Cell. Biochem. 13, 165-192

Miller, E.J., Epstein, E.H. & Piez, K.A. (1971) Biochem. Biophys. Res. Commun. 42, 1024-1029

Miller, E.J. & Matukas, V.J. (1969) Proc. Natl. Acad. Sci. U.S.A. 64, 1264-1268

Montfort, I. & Pérez-Tamayo, R. (1975) Connect. Tissue Res. 3, 245-252

Montfort, I. & Pérez-Tamayo, R. (1975) J. Histochem. Cytochem. 23, 910-920

Moxham, B.J. & Berkovitz, R.K.B. (1974) Arch. Oral Biol. 19, 903-909

Muir, H. & Hardingham, T.E. (1975) in MTP Intern. Rev. Sci. Biochemistry Series One, Biochemistry of Carbohydrates (Whelan, W.J., ed.), vol. 5, pp. 153-222, Butterworth's, London

Munemoto, K., Iwayama, Y., Yoshida, M., Sera, M., Aono, M. & Yokomizo, I. (1970) Arch. Oral Biol. 15, 369-382

Myers, D.B., Highton, T.C. & Rayns, D.G. (1973) J. Ultrastruct. Res. 42, 87-92

McDevitt, C.A. & Muir, H. (1971) Anal. Biochem. 44, 612-622

McDuffie, N.M., Dietrich, C.P. & Nader, H.B. (1975) Biopolymers 14, 1473-1486

Nakashima, Y., Di Ferrante, N., Jackson, R.L. & Pownall, H.J. (1975) J. Biol. Chem. 250, 5386-5392

Ness, A.R. (1967) in The Mechanisms of Tooth Support, pp. 84-88, Wright, Bristol

Neuberger, A., Gottschalk, A. & Marshall, R.D. (1966) in Glycoproteins. Their Composition, Structure and Function (Gottschalk, A., ed.), pp. 273-296, Elsevier, Amsterdam

O'Brian,C., Bhaskar, S.H. & Brodie, A.G. (1958) J. Dent. Res. 37, 467-484

Öbrink, B. (1972) Biochim. Biophys. Acta 264, 354-361

Öbrink, B. (1973) Eur. J. Biochem. 33, 387-400

Öbrink, B. (1975) in Structure of Fibrous Biopolymers (Atkins, E.D.T. & Keller, A., eds.), pp. 81-92, Butterworth's, London

Öbrink, B. & Sundelöf, L.-O. (1973) Eur. J. Biochem. 37, 226-232

Öbrink, B. & Wasteson, Å. (1971) Biochem. J. 121, 227-233

Oegema, T.R., Hascall, V.C. & Dziewiatkowski, D.D. (1975a) J. Biol. Chem. 250, 6151-6159

Oegema, T.R., Laidlaw, J., Hascall, V. & Dziewiatkowski, D.D. (1975b) Arch. Biochem. Biophys. 170, 698-709

Ogston, A.G. (1970) in Chemistry and Molecular Biology of the Intercellular Matrix (Balazs, E.A., ed.), pp. 1231-1240, Academic Press, New York

Ogston, A.G. & Sherman, T.F. (1959) Biochem. J. 72, 301-305

Olivecrona, T., Bengtsson, G., Marklund, S.E., Lindahl, U. & Höök, M. (1977), Fed. Proc. 36, 60-65

Orban, B. (1928) J. Am. Dent. Assoc. 15, 1004-1016

Pardo, A. & Peréz-Tamayo, R. (1975) Biochim. Biophys. Acta 392, 121-130

Parfitt, G.J. (1960) J. Dent. Res. 39, 608-618

Parfitt, G.J. (1967) in The Mechanisms of Tooth Support (Anderson, D.J., Eastoe, J.E., Melcher, A.H. & Picton, D.C.A., eds.), pp. 154-156, Wright, Bristol

Pasternack, S.G., Veis, A. & Breen, M. (1974) J. Biol. Chem. 249, 2206-2211

Paunio, K. (1969) Suom. Hammaslääk. Toim. 65, 249-290

Pearson, C.H., Ainsworth, L. & Chovelon, A. (1978a) Connect. Tissue Res. 6, 51-59

Pearson, C.H., Davies, J.D., Gibson, G.J., Lehocky, S. & Scott, P.G. (1978b) Biochem. Soc. Trans., in press

Pearson, C.H., Wohllebe, M., Carmichael, D.J. & Chovelon, A. (1975) Connect. Tissue Res. 3, 195-206

Pearson, J.P. & Mason, R.M. (1977) Biochim. Biophys. Acta 498, 176-188

Piez, K.A., Eigner, E.A. & Lewis, M.S. (1963) Biochemistry 2, 58-66

Poole, A.R., Barratt, M.E.J. & Fell, H.B. (1973) Int. Arch. Allergy 44, 469-488

Pratt, R.M., Larsen, M.A. & Johnston, M.C. (1975) Dev. Biol. 44, 298-305

Preston, B.N. (1968) Arch. Biochem. Biophys. 126, 974-976

Radhakrishnamurthy, B., Ruiz, H.A. & Berenson, G.S. (1977) J. Biol. Chem. 252, 4831-4841

Reddi, A.H., Hascall, V.C. & Hascall, G.K. (1978) J. Biol. Chem. 253, 2429-2436

Rees, D.A. (1975) in MTP Intern. Rev. Sci. Biochemistry Series one, Biochemistry of Carbohydrates (Whelan, W.J., ed.), pp. 1-42, Butterworth's, London

Robinson, H.C. & Hopwood, J.J. (1973) Biochem. J. 133, 457-470

Rodén, L. & Schwartz, N.B. (1975) in MTP Intern. Rev. Sci. Biochemistry Series One, Biochemistry of Carbohydrates (Whelan, W.J., ed.), pp.95-152, Butterworth's, London

Rodén, L. & Smith, R. (1966) J. Biol. Chem. 241, 5949-5954

Rosenberg, L., Hellmann, W. & Kleinschmidt, A.K. (1975) J. Biol. Chem. 250, 1877-1883

Rosenberg, L., Wolfenstein-Todel, C., Margolis, R., Pal, S. & Stridor, W. (1976) J. Biol. Chem. 251, 6439-6444

Rossman, L.E., Rosenbloom, S. & Robinson, P. (1975) J. Dent. Res. 54, 1115-1119

Roughley, P.J. (1977) Biochem. J. 167, 639-646

Roughley, P.J. & Barrett, A.J. (1977) Biochem. J. 167, 629-637

Saito, H., Yamagata, T. & Suzuki, S. (1968) J. Biol. Chem. 243, 1536-1542

Sajdera, S.W. & Hascall, V.C. (1969) J. Biol. Chem. 244, 77-87

Saunders, R.L. de C.H. & Röckert, H.O. (1967) in Structural and Chemical Organization of Teeth (Miles, A.E.W., ed.), pp.199-245, Academic Press, New York

Schaffrath, D., Stuhlsatz, H.W. & Greiling, H. (1976) Hoppe-Seyler's Z. Physiol. Chem. 357, 499-508

Scher, I. & Hamerman, D. (1972) Biochem. J. 126, 1073-1080

Schuel, H., Kelly, J.W., Berger, E.R. & Wilson, W.L. (1974) Exp. Cell Res. 88, 24-30

Scott, J.E., (1960) in Methods of Biochemical Analysis (Glick, D., ed.), Vol. 8, pp. 145-198, Interscience, New York

Scott, J.E. & Dorling, J. (1965) Histochemie 5, 221-223

Scott, J.H. & Symons, N.B.B. (1971) Introduction to Dental Anatomy, Livingstone, Edinburgh

Scott, P.G. & Pearson, C.H. (1978) FEBS Lett. 88, 41-45

Shackleford, J.M. (1971) J. Periodont. Res. 6, 45-54

Sheehan, J.K., Nieduszynski, I.A., Phelps, C.F., Muir, H. & Hardingham, T.E. (1978) Biochem. J. 171, 109-114

Sicher, H. & Bhaskar, S.N. (1972) Orban's Oral Histology and Embryology, Mosby, St. Louis

Siegel, R.C., Pinnell, S.R. & Martin, G.R. (1970) Biochemistry 9, 4486-4492

Silpananta, P., Dunstone, J.R. & Ogston, A.G. (1968) Biochem. J. 109, 43-50

Smith, Q.T. & Lindenbaum, A. (1971) Calcif. Tissue Res. 7, 290-298

Sodek, J. (1977) Arch. Oral Biol. 22, 655-665

Sodek, J., Brunette, D.M., Feng, J., Heersche, J.N.M., Limeback, H.F., Melcher, A.H. & Ng, B. (1977) Arch. Oral Biol. 22, 647-653

Spooner, B.S. & Wessells, N.K. (1970) Proc. Natl. Acad. Sci. U.S.A. 66, 360-361

Srinivasan, S.R., Radhakrishamurthy, B. & Berenson, G.S. (1975) Arch. Biochem. Biophys. 170, 334-340

Stanescu, V. & Maroteaux, P. (1975) Pediat. Res. 9, 779-782

Stark, G.R., Stein, W.H. & Moore, S. (1960) J. Biol. Chem. 235, 3177-3181

Starkey, P.M. & Barrett, A.J. (1973) Biochem. J. 131, 823-831

Starkey, P.M. & Barrett, A.J. (1976) Biochem. J. 155, 255-263

Stegeman, H., & Stalder, K. (1967) Clin. Chim. Acta 18, 267-273

Stein, G.S., Roberts, R.M., Davis, J.L., Head, W.J., Stein, J.L., Thrall, C.L., Van Veen, J. & Welch, D.W. (1975) Nature 258, 639-641

Stern, E.L., Lindahl, B. & Róden, L. (1971) J. Biol. Chem. 246, 5707-5715

Suzuki, S. (1960) J. Biol. Chem. 235, 3580-3586

Swann, D.A., Powell, S., Broadhurst, J., Sordillo, E. & Sotman, S. (1976) Biochem. J. 157, 503-506

Synge, J.L. (1933) Phil. Trans. R. Soc. Series A 231, 437-477

Tanaka, Y. & Gore, I. (1966) J. Chromatogr. 23, 254-260

Teien, A.N., Abildgaard, U. & Höök, M. (1976) Thromb. Res. 8, 859-867

Ten Cate, A.R. (1969) in Biology of the Periodontium (Melcher, A.H. & Bowen, N.H., eds.), pp. 53-90, Academic Press, London

Ten Cate, A.R., Deporter, D.A. & Freeman, E. (1976) Am. J. Orthod. 69, 155-168

Terho, T.T. & Hartiala, K. (1971) Anal. Biochem. 41, 471-476

Thomas, N.R. (1965) J. Dent. Res. 44, 1159

Thomas, N.R. (1976) in The Eruption and Occlusion of Teeth (Poole, D.F.G. & Stack, M.V., eds.), pp. 290-309, Butterworth's, London

Thyberg, J., Lohmander, S. & Heinegård, D. (1975) Biochem. J. 151, 157-166

Toole, B.P. (1972) Dev. Biol. 29, 321-329

Toole, B.P. (1973) Amer. Zool. 13, 1061-1065

Toole, B.P. (1976) J. Biol. Chem. 251, 895-897

Toole, B.P. & Gross, J. (1971) Dev. Biol. 25, 57-77

Toole, B.P. & Lowther, D.A. (1965) Biochim. Biophys. Acta 101, 364-366

Toole, B.P. & Lowther, D.A. (1966) Biochim. Biophys. Acta 121, 315-325

Toole, B.P. & Lowther, D.A. (1968a) Biochem. J. 109, 857-866

Toole, B.P. & Lowther, D.A. (1968b) Arch. Biochem. Biophys. 128, 567-578

Toole, B.P. & Trelstad, R.L. (1971) Dev. Biol. 28, 28-35

Trelstad, R.L. (1974) Biochem. Biophys. Res. Commun. 57, 717-725

Tsiganos, C.P. & Muir, H. (1967) Biochem. J. 104, 26c-28c

Urist, M.R., Speer, D.P., Ibsen, K.J. & Strates, B.S. (1968) Calcif. Tissue Res. 2, 253-261

Uvnäs, B. (1974) Life Sci. 14, 2355-2366

Uvnäs, B. & Åborg, C.-H. (1976) Acta Physiol. Scand. 96, 512-525

Waldman, A.A., Marx, G. & Goldstein, J. (1974) Biochim. Biophys. Acta 343, 324-329

Wasteson, Å. (1969) Biochim. Biophys. Acta 177, 152-154

Wasteson, Å. (1971) J. Chromatogr. 59, 87-97

Weibkin, O.W. & Muir, H. (1973) FEBS lett. 37, 42-46

Wessells, N.K., Spooner, B.S., Ash, J.F., Bradley, M.O., Luduena, M.A., Taylor, E.L., Wrenn, J.T. & Yamada, K.M. (1971) Science 171, 135-143

Wills, D.J., Picton, D.C.A. & Davies, W.I.R. (1972) J. Periodont. Res. 7, 42-51

Wood, G.C. & Keech, M.K. (1960) Biochem. J. 75, 588-598

Yamagata, T., Saito, H., Habauchi, O. & Suzuki, S. (1968) J. Biol. Chem. 243, 1523-1535

Yamanishi, H. & Sato, Y. (1976) J. Biochem. 79, 131-144

Yphantis, D.A. (1964) Biochemistry 3, 297-317